

(19) Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11) EP 0 870 841 A1

(12) **EUROPEAN PATENT APPLICATION**  
published in accordance with Art. 158(3) EPC

(43) Date of publication:  
14.10.1998 Bulletin 1998/42

(51) Int. Cl.<sup>6</sup>: C12P 19/26, C12N 1/21,  
C12N 15/54, C12N 5/16  
// (C12P19/26, C12R1:19, 1:15)

(21) Application number: 97940365.6

(86) International application number:  
PCT/JP97/03226

(22) Date of filing: 12.09.1997

(87) International publication number:  
WO 98/12343 (26.03.1998 Gazette 1998/12)

(84) Designated Contracting States:  
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC  
NL PT SE

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(30) Priority: 17.09.1996 JP 244451/96  
28.10.1996 JP 285066/96

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(54) **PROCESSES FOR PRODUCING SUGAR NUCLEOTIDES AND COMPLEX CARBOHYDRATES**

(57) This invention relates to a process for producing a sugar nucleotide, in which a) a culture broth of a microorganism capable of producing NTP from a nucleotide precursor, or a treated product of the culture broth, and b) a culture broth of a microorganism capable of producing a sugar nucleotide from a sugar and NTP, or a treated product of the culture broth, are used as enzyme sources; a process for producing a complex carbohydrate, in which the above-described a) and b) and c) a culture broth of a microorganism, an animal cell or an insect cell capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor, or a treated product of the culture broth, are used as enzyme sources; a process for producing a complex carbohydrate, in which a culture broth of a microorganism, an animal cell or an insect cell capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor, or a treated product of the culture broth, is used as an enzyme source; and a process for producing N-acetylglucosamine-1-phosphate, in which a culture broth of a microorganism having strong galactokinase activity, or a treated product of the culture broth, is used as the enzyme source.

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**Description****TECHNICAL FIELD**

5 This invention relates to a process for producing a complex carbohydrate which is useful for protection against infection of bacteria, viruses and the like, application to cardiovascular disorders and immunotherapy and to a process for producing a sugar nucleotide which is important as a substrate for the synthesis of the complex carbohydrate.

**BACKGROUND ART**

10 Examples of the known process for producing sugar nucleotides include: 1) chemical synthetic processes (*Adv. Carbohydr. Chem. Biochem.*, 28, 307 (1973), *Bull. Chem. Soc. Japan*, 46, 3275 (1973), *J. Org. Chem.*, 57, 146 (1992), *Carbohydr. Res.*, 242, 69 (1993)); 2) production processes using enzymes (*J. Org. Chem.*, 55, 1834 (1990), *J. Org. Carbohydr. Res.*, 242, 69 (1993)); 3) processes using microbial cells such as yeast and the like (Japanese Published Examined Patent Application No. 2073/70, Japanese Published Examined Patent Application No. 40756/71, Japanese Published Examined Patent Application No. 1837/72, Japanese Published Examined Patent Application No. 26703/72, Japanese Published Examined Patent Application No. 8278/74, Japanese Published Unexamined Patent Application No. 268692/90); and 4) an extraction process from microbial cells of halo-tolerant yeast (Japanese Published Unexamined Patent Application No. 23993/96).

15 20 However, the process 1) requires expensive materials (for example, morpholidate derivative of nucleotide-5'-monophosphate (referred to as "NMP" hereinafter), sugar phosphate, etc.); the process 2) requires expensive materials (for example, nucleotide-5'-diphosphate (referred to as "NDP" hereinafter), nucleotide-5'-triphosphate (referred to as "NTP" hereinafter), phosphoenolpyruvate, etc.), and various enzymes (e.g., pyruvate kinase, etc.); and the process 3) requires drying treatment of microbial cells. Including the process 4), all of the above-mentioned processes use expensive nucleotides, sugar phosphates, and the like or have a difficulty in effecting large scale production from the operational point of view, so that an industrial scale production process of sugar nucleotides has not so far been established.

25 Examples of the known process for producing complex carbohydrates include 1) chemical synthetic processes (Method in Enzymol., 247, 193 (1994), *Angew. Chem. Int. Ed. Engl.*, 21, 155 (1982), *Carbohydr. Res.*, 211, c1 (1991)), 2) processes in which a hydrolase is used (*Anal. Biochem.*, 202, 215 (1992), *Trends Biotechnol.*, 6, 256 (1988)) and 3) processes in which a glycosyltransferase is used (Japanese Published Unexamined Patent Application No. 79792/95, Japanese Published National Publication No. 500248/95, Japanese Published Examined Patent Application No. 82200/93, WO 94/25614, Japanese Published National Publication No. 503905/97, U.S. Patent 5,583,042).

30 35 The introduction of protecting groups is essential for stereo-selective synthesis in the process 1). The yield and selectivity are not sufficient in the process 2). Expensive materials (for example, NDP, NTP, phosphoenolpyruvic acid, sugar phosphate, sugar nucleotide, etc.) and various enzymes (for example, pyruvate kinase, etc.) are necessary in the process 3). Therefore, these processes have not been established as inexpensive industrial production processes of complex carbohydrates. In addition, there has been nothing known about a process for the direct industrial production of complex carbohydrates, which uses only inexpensive nucleotide precursors, sugars and complex carbohydrate precursors as the starting materials.

40 45 It has been reported that UMP is produced in a microorganism belonging to the genus *Corynebacterium* when orotic acid is added (Amino Acid, Nucleic Acid, 23, 107 (1971)). In addition, a process in which cytidine diphosphate choline is formed from orotic acid as the material is also known (Japanese Published Unexamined Patent Application No. 276974/93).

**DISCLOSURE OF THE INVENTION**

An object of the present invention is to provide a process for producing a complex carbohydrate which is useful for protection against infection of bacteria, viruses, and the like, application to cardiovascular disorders and immunotherapy, and a process for producing a sugar nucleotide which is important as a substrate for synthesizing the complex carbohydrate at a low cost and efficiently.

50 55 The inventors of the present invention have conducted intensive studies on the microbial production of a complex carbohydrate and a sugar nucleotide using a nucleotide precursor as the starting material, and have found as the results that a sugar nucleotide can be produced by using only a nucleotide precursor and a sugar as the materials, that productivity of the sugar nucleotide can be improved by strengthening expression of genes involved in its biosynthesis and that a complex carbohydrate can be produced efficiently, using a microorganism capable of producing the sugar nucleotide and a microorganism, an animal cell or an insect cell capable of producing the complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor and using a nucleotide precursor, a sugar and a complex car-

bohydrate precursor as the only starting materials, thereby resulting in the accomplishment of the present invention.

The present invention provides a process for producing a complex carbohydrate, which comprises: selecting, as enzyme sources, a) a culture broth of a microorganism capable of producing NTP from a nucleotide precursor, or a treated product of the culture broth, b) a culture broth of a microorganism capable of producing a sugar nucleotide from a sugar and NTP, or a treated product of the culture broth, and c) a culture broth of a microorganism, an animal cell or an insect cell capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor, or a treated product of the culture broth; allowing the enzyme sources, the nucleotide precursor, the sugar and the complex carbohydrate precursor to be present in an aqueous medium to form and accumulate the complex carbohydrate in the aqueous medium; and recovering the complex carbohydrate from the aqueous medium, a process for producing a sugar nucleotide, which comprises: selecting, as enzyme sources, a) a culture broth of a microorganism capable of producing NTP from a nucleotide precursor, or a treated product of the culture broth, and b) a culture broth of a microorganism capable of producing a sugar nucleotide from a sugar and NTP, or a treated product of the culture broth; allowing the enzyme sources, the nucleotide precursor and the sugar to be present in an aqueous medium to form and accumulate the sugar nucleotide in the aqueous medium; and recovering the sugar nucleotide from the aqueous medium, and a process for producing a complex carbohydrate, which comprises: selecting, as an enzyme source, a culture broth of a microorganism, an animal cell or an insect cell capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor obtained by the above-described process for producing a sugar nucleotide and the complex carbohydrate precursor to be present in an aqueous medium to form and accumulate the complex carbohydrate in the aqueous medium; and recovering the complex carbohydrate from the aqueous medium. It also provides a process for producing N-acetylglucosamine-1-phosphate, which comprises selecting, as an enzyme source, a culture broth of a microorganism having strong galactokinase activity, or a treated product of the culture broth; allowing the enzyme source and N-acetylglucosamine to be present in an aqueous medium to form and accumulate N-acetylglucosamine-1-phosphate in the aqueous medium; and recovering the N-acetylglucosamine 1-phosphate from the aqueous medium.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows construction steps of expression plasmids pPA31 and pPAC31.

Fig. 2 shows construction steps of galU, ppa gene expression plasmids pNT12 and pNT32.

30 Fig. 3 shows construction steps of galT, galK gene expression plasmid pNT25.

Fig. 4 shows construction steps of plasmid pTK7 which expresses galT and galK genes in *Corynebacterium ammoniagenes*.

Fig. 5 shows construction steps of glmU, ppa gene expression plasmid pNT14.

Fig. 6 shows construction steps of pgm gene expression plasmid pNT24.

35 Fig. 7 shows construction steps of glmM gene expression plasmid pNT44.

Fig. 8 shows construction steps of glk gene expression plasmid pNT46.

Fig. 9 shows construction steps of pfkB gene expression plasmid pNT47.

Fig. 10 shows construction steps of galK gene expression plasmid pNT54.

Fig. 11 shows construction steps of manB, manC gene expression plasmid pNK7.

40 Fig. 12 shows construction steps of pgm, pfkB gene expression plasmid pNT55.

Fig. 13 shows construction steps of gmd, wcaG gene expression plasmid pNK8.

Fig. 14 shows construction steps of neuA gene expression plasmid pTA14.

Fig. 15 shows construction steps of lgtC gene expression plasmid pGT3.

45 Fig. 16 shows construction steps of lgtB gene expression plasmid pNT60.

Abbreviations to be used herein and description of the abbreviations are shown in Table 1-(1) and Table 1-(2).

Table 1- (1)

Glc	glucose
G-6-P	glucose-6-phosphate
G-1-P	glucose-1-phosphate
Glc-1,6-P2	glucose-1,6-diphosphate
Gal	galactose
Gal-1-P	galactose-1-phosphate
GlcN-6-P	glucosamine-6-phosphate

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Table 1- (1) (continued)

5	GlcN-1-P	glucosamine-1-phosphate
	GlcUA	glucuronic acid
	GlcN	glucosamine
	GlcNAc	N-acetylglucosamine
	GlcNAc-1-P	N-acetylglucosamine-1-phosphate
10	F-6-P	fructose-6-phosphate
	F-1,6-P2	fructose-1,6-diphosphate
	Man	mannose
	Man-6-P	mannose-6-phosphate
15	Man-1-P	mannose-1-phosphate
	GDP-4-keto-6-deoxyMan	guanosine-5'-diphospho-4-keto-6-deoxymannose
	ManNAc	N-acetylmannosamine
	NeuAc	N-acetylneurameric acid
20	acetyl CoA	acetyl coenzyme A
	NTP	nucleotide-5'-triphosphate
	NDP	nucleotide-5'-diphosphate
25	NMP	nucleotide-5'-monophosphate
	ATP	adenosine-5'-triphosphate
	UTP	uridine-5' -triphosphate
	GTP	guanosine-5'-triphosphate
30	CTP	cytidine-5'-triphosphate
	GMP	guanosine-5'-monophosphate

35

Table 1-(2)

40	UDP-Glc	uridine-5'-diphosphoglucose
	UDP-Gal	uridine-5'-diphosphogalactose
	UDP-GlcNAc	uridine-5'-diphospho-N-acetylglucosamine
	UDP-GalNAc	uridine-5'-diphospho-N-acetylgalactosamine
45	UDP-GlcUA	uridine-5'-diphosphoglucuronic acid
	GDP-Man	guanosine-5'-diphosphomannose
	GDP-Fuc	guanosine-5'-diphosphofucose
	CMP-NeuAc	cytidine-5'-monophospho-N-acetylneurameric acid
50	galU	glucose-1-phosphate uridyltransferase
	ppa	(inorganic) pyrophosphatase
	galK	galactokinase
55	galT	galactose-1-phosphate uridyltransferase
	glmU	N-acetylglucosamine-1-phosphate uridyltransferase glucosamine-1-phosphate acetyltransferase
	pgm	phosphoglucomutase

Table 1-(2) (continued)

5	pfkB	phosphofructokinase
	glmM	phosphoglucosamine mutase
	glk	glucokinase
	manB	phosphomannomutase
	manC	mannose-1-phosphate guanyltransferase
10	gmd	GDP-mannose-4,6-dehydratase
	wcaG	GDP-4-keto-6-deoxymannose epimerase/reductase
	neuA	CMP-N-acetylneurameric acid synthetase
	neuB	N-acetylneurameric acid synthetase
15	nanA	N-acetylneurameric acid aldolase
	pyrG	cytidine-5'-triphosphate synthetase
	lgtB	$\beta$ 1,4-galactosyltransferase
20	lgtC	$\alpha$ 1,4-galactosyltransferase
	ugd	UDP-glucose dehydrogenase

According to the present invention, a novel production process of a sugar nucleotide and a novel production process of a complex carbohydrate using the sugar nucleotide production process can be provided, which are characterized in that 1) expensive materials (for example, NTP, sugar phosphates, etc.) are not required, and inexpensive nucleotide and a sugar can be used as the sole starting materials, 2) addition of expensive phosphoenolpyruvic acid and pyruvate kinase is not necessary in converting NMP or NDP into NTP, and 3) a process for the isolation of enzymes is not necessary.

With regard to the sugar nucleotide to be produced by the production process of the present invention, compounds having a general structure in which the terminal phosphate group of a nucleoside 5'-diphosphate residue and the reducing group of a sugar residue are linked together by ester bonding can be exemplified, and those compounds in which the nucleotide residue is cytidine 5'-monophosphate and the sugar residue is a polyol are also included in the sugar nucleotide to be produced by the present invention.

Examples of the complex carbohydrate to be produced by the production process of the present invention include compounds in which carbohydrates are bound to monosaccharides, oligosaccharides, monosaccharides or oligosaccharides linked to a carrier or the like, proteins, peptides, lipids, glycoproteins, glycolipids, glycopeptides, steroid compounds or the like.

The present invention will be described in detail below.

1) With regard to the microorganism for use in the present invention capable of producing NTP from a nucleotide precursor, any microorganism capable of producing NTP from a nucleotide precursor can be used. Examples include microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium*.

The microorganisms belonging to the genus *Escherichia* include *Escherichia coli* and the like.

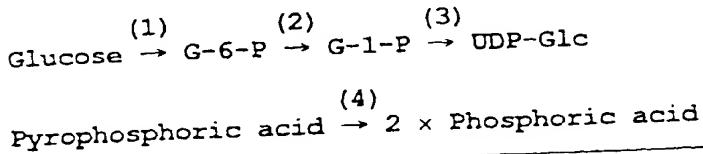
The microorganisms belonging to the genus *Corynebacterium* include *Corynebacterium ammoniagenes* and the like.

2) As the microorganism for use in the present invention capable of producing a sugar nucleotide from a sugar and NTP, any microorganism having the activity to form the sugar nucleotide of interest can be used as follows.

2) - (i) With regard to the production of UDP-Glc, it is preferred to use a microorganism having strong enzyme activities of (1) to (4) shown in the following formula 1.

Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

In addition, a transformant in which at least one of the enzyme activities selected from (1), (2), (3) and (4) are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* KY8415 (FERM BP-408) having recombinant DNA (pNT12) which contains galU and ppa genes derived from *Escherichia coli*, and the like.



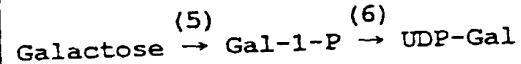
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(1): Hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2)  
 (2): Phosphoglucomutase (EC 2.7.5.1)  
 15 (3): Glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)  
 (4): (Inorganic) pyrophosphatase (EC 3.6.1.1)

20 2) - (ii) With regard to the production of UDP-Gal, it is preferred to use a microorganism having strong enzyme activities of (5) and (6) shown in the following formula 2, preferably further having strong enzyme activities of (1) to (4) shown in the above-mentioned formula 1.

25 Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*. In addition, a transformant in which at least one of the enzyme activities selected from (5) and (6), or at least one of the enzyme activities selected from (5) and (6) and at least one of the enzyme activities selected from (1) to (4), are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* NM522 having recombinant DNA (pNT25) which contains galT and galK genes derived from *Escherichia coli* and *Corynebacterium ammoniagenes* ATCC 21170 having recombinant DNA (pTK7) which contains galT and galK genes derived from *Escherichia coli*.

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(Formula 2)

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40 (5): Galactokinase (EC 2.7.1.6)  
 (6): Galactose-1-phosphate uridylyltransferase (EC 2.7.7.12)

2) - (iii) With regard to the production of UDP-GlcNAc, it is preferred to use a microorganism having strong enzyme activities of (7) to (12) shown in the following formula 3 and having a strong enzyme activity of (4) shown in formula 1, or a microorganism having strong enzyme activities of (13) and (10) shown in formula 3.

45 Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

50 In addition, a transformant in which at least one of the enzyme activities selected from (4), (7), (8), (9), (10) and (13) are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* NM522 having recombinant DNA (pNT44) which contains galmM gene derived from *Escherichia coli*, *Escherichia coli* KY8415 having recombinant DNA (pNT14) which contains glmU and ppa genes derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT46) which contains glk gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT54) which contains galK gene derived from *Escherichia coli*, and the like.

55 Although it is necessary to add Glc-1,6-P2 for the expression and increment of the phosphoglucosamine mutase activity of (8) (*J. Biol. Chem.*, 271, 32 (1996)), it is possible to provide Glc-1,6-P2 from G-6-P and F-6-P without adding Glc-1,6-P2 by using a transformant in which the enzyme activities of (11) and (12) are increased by recombinant DNA techniques.

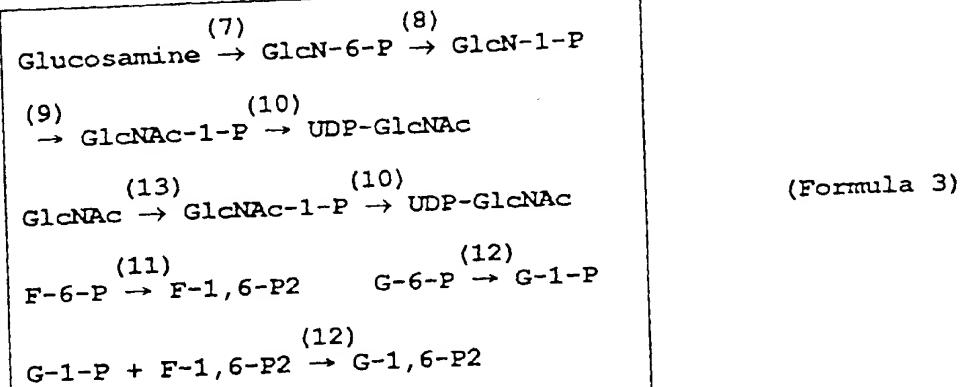
Specific examples of such a transformant include *Escherichia coli* NM522 having recombinant DNA

(pNT24) which contains *pgm* gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT47) which contains *pfkB* gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT55) which contains *pgm* and *pfkB* gene derived from *Escherichia coli*, and the like.

The process in which expression of the phosphoglucosamine mutase activity of (8) is increased by providing Glc-1,6-P2 from G-6-P and F-6-P using the enzyme activities of (11) and (12) is a process disclosed for the first time by the present invention.

The process in which GlcNAc-1-P is produced from GlcNAc using the galactokinase (EC 2.7.1.6) of (13) is a process disclosed for the first time by the present invention. It is possible to produce GlcNAc-1-P by using the process. That is, GlcNAc-1-P can be produced by using a culture broth or a treated product of the culture broth of a microorganism having strong galactokinase activity, such as a microorganism which contains recombinant DNA consisting of a DNA fragment containing a *galK*-encoding gene and a vector, as an enzyme source, allowing the enzyme source and GlcNAc to be present in an aqueous medium to form and accumulate GlcNAc-1-P in the aqueous medium, and recovering the thus-obtained GlcNAc-1-P from the aqueous medium.

Recovery of GlcNAc-1-P from the aqueous medium can be carried out in the usual way in which activated carbon, an ion exchange resin, and the like are used.



(7): Hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2)

(8): Phosphoglucosamine mutase

(9): Glucosamine-1-phosphate acetyltransferase

(10): N-Acetylglucosamine-1-phosphate uridyltransferase (EC 2.7.7.23)

(11): Phosphofructokinase (EC 2.7.1.11)

(12): Phosphoglucomutase (EC 2.7.5.1)

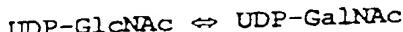
(13): Galactokinase (EC 2.7.1.6)

2)-(iv) With regard to the production of UDP-GalNAc, it is preferred to use a microorganism having strong enzyme activities of (7) to (12) shown in formula 3, of (14) shown in formula 4 and of (4) shown in formula 1, or a microorganism having strong enzyme activities of (10) and (13) shown in formula 4 and of (14) shown in formula 4.

Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

In addition, a transformant in which at least one of the enzyme activities selected from (7) to (14) and (4) are increased by recombinant DNA techniques can also be used.

(14)



(Formula 4)

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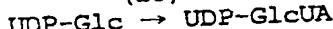
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(14): UDP-GlcNAc 4-epimerase (EC 5.1.3.7)

2)-(v) With regard to the production of UDP-GlcUA, it is preferred to use a microorganism having strong enzyme activities of (1) to (4) shown in formula 1 and of (15) shown in formula 5. Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*. In addition, a transformant in which at least one of the enzyme activities selected from (1), (2), (3), (4) and (15) are increased by recombinant DNA techniques can also be used.

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(15)



(Formula 5)

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(15): UDP-Glc dehydrogenase (EC 1.1.1.22)

2) - (vi) With regard to the production of GDP-Man, it is preferred to use a microorganism having strong enzyme activities of (16) to (18) shown in the following formula 6 and of (11) and (12) shown in formula 3. Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

35

In addition, a transformant in which at least one of the enzyme activities selected from (16), (17) and (18) are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* NM522 having recombinant DNA (pNK7) which contains manB and manC genes derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT46) which contains glk gene derived from *Escherichia coli*, and the like.

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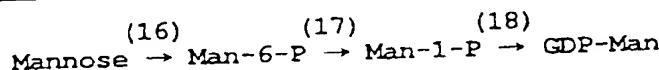
Although it is necessary to add Glc-1,6-P2 for the expression and increment of the phosphomannomutase activity of (17) by recombinant DNA techniques, it is possible to provide Glc-1,6-P2 from G-6-P and F-6-P without adding Glc-1,6-P2 by using a transformant in which the enzyme activities of (11) and (12) are increased by recombinant DNA techniques. Specific examples of such a transformant include *Escherichia coli* NM522 having recombinant DNA (pNT24) which contains pgm gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT47) which contains pfkB gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT55) which contains pgm and pfkB genes derived from *Escherichia coli*, and the like.

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The process in which expression of the phosphomannomutase activity of (17) is increased by providing Glc-1,6-P2 from G-6-P and F-6-P using the enzyme activities of (11) and (12) is a process disclosed for the first time by the present invention.

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(Formula 6)

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(16): Hexokinase (EC 2.7.1.1) or glucokinase (EC 2.7.1.2)

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(17): Phosphomannomutase (EC 2.7.5.7)

20

(18): Mannose-1-phosphate guanyltransferase (EC 2.7.7.13)

2)- (vii) With regard to the production of GDP-Fuc, it is preferred to use a microorganism having strong enzyme activities of (19) and (20) shown in the following formula 7, of (16) to (18) shown in formula 6 and of (11) and (12) shown in formula 3.

25

Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

30

In addition, a transformant in which at least one of the enzyme activities selected from (16), (17), (18), (19) and (20) are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* NM522 having recombinant DNA (pNK7) which contains manB and manC genes derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNK8) which contains gmd and wcaG genes derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT46) which contains glk gene derived from *Escherichia coli*, and the like.

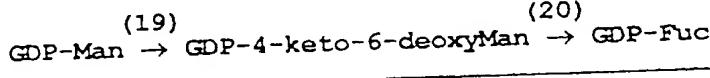
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Although it is necessary to add Glc-1,6-P2 for the expression and increment of the phosphomannomutase activity of (17) by recombinant DNA techniques, it is possible to provide Glc-1,6-P2 from G-6-P and F-6-P without adding Glc-1,6-P2 by using a transformant in which the enzyme activities of (11) and (12) are increased by recombinant DNA techniques.

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Specific examples of such a transformant include *Escherichia coli* NM522 having recombinant DNA (pNT24) which contains pgm gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT47) which contains pfkB gene derived from *Escherichia coli*, *Escherichia coli* NM522 having recombinant DNA (pNT55) which contains pgm and pfkB genes derived from *Escherichia coli*, and the like.

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(Formula 7)

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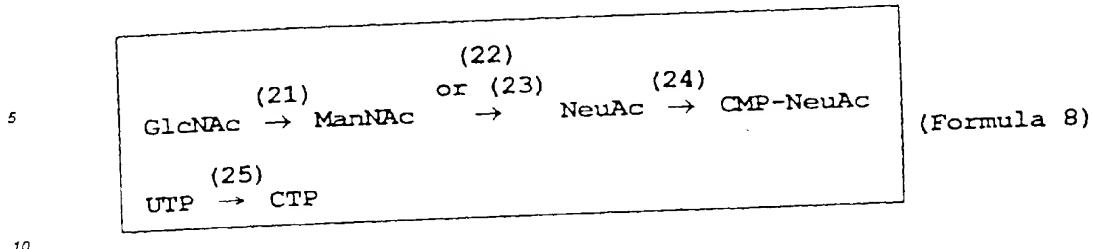
(19): GDP-Man-4,6-dehydratase (EC 4.2.1.47)  
 (20): GDP-4-keto-6-deoxymannose epimerase/reductase

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2) - (viii) With regard to the production of CMP-NeuAc, it is preferred to use a microorganism having strong enzyme activities of (21), (22) or (23), (24) and (25) shown in the following formula 8.

Specifically, microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium* can be exemplified. Specific examples include *Escherichia coli* and *Corynebacterium ammoniagenes*.

In addition, a transformant in which at least one of the enzyme activities selected from (21), (22), (23), (24) and (25) are increased by recombinant DNA techniques can also be used. Specific examples of the transformant include *Escherichia coli* C600 having recombinant DNA (pNAL1) which contains nanA gene derived from *Escherichia coli* (*Appl. Environ. Microbiol.*, 51, 562 (1986)), *Escherichia coli* NM522 having recombinant DNA (pTA14) which contains neuA gene derived from *Escherichia coli*, and the like.



15

- (21): GlcNAc 2-epimerase (EC 5.1.3.8)
- (22): NeuAc aldolase (EC 4.1.3.3)
- (23): NeuAc synthetase (EC 4.1.3.19)
- (24): CMP-NeuAc synthetase (EC 2.7.7.43)
- (25): CTP synthetase (EC 6.3.4.2)

20 When a microorganism has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2), it is possible to produce a sugar nucleotide from a nucleotide precursor and a sugar using the microorganism.

25 It is possible to produce UDP-Glc from a UTP precursor such as orotic acid or the like and glucose using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(i); UDP-Gal from a UTP precursor such as orotic acid or the like and galactose using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(ii); UDP-GlcNAc from a UTP precursor such as orotic acid or the like and glucosamine or N-acetylglucosamine using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(iii); UDP-GalNAc from a UTP precursor such as orotic acid or the like and glucosamine or N-acetylglucosamine using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(iv); UDP-GlcUA from a UTP precursor such as orotic acid or the like and glucose using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(v); GDP-Man from a GTP precursor such as GMP or the like and mannose using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(vi); GDP-Fuc from a GTP precursor such as GMP or the like and mannose using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(vii); and CMP-NeuAc from a CTP precursor such as orotic acid or the like and N-acetylglucosamine or N-acetylmannosamine using a microorganism which has both of the properties of microorganisms described in 1) and the properties of microorganisms described in 2)-(viii).

40 Specific examples of such microorganism include *Corynebacterium ammoniagenes* capable of expressing galT and galK genes derived from *Escherichia coli*.

45 Unlike the case of the above-mentioned strain, when a single strain has only a part of activities required for producing a sugar nucleotide, the sugar nucleotide can be produced by optionally combining microorganisms having respective activities.

50 The properties described in 1) are not necessarily owned by a single microorganism, and two or more microorganisms in which the properties described in 1) are independently located can also be used as the microorganism having the properties described in 1). Specifically, a combination of *Escherichia coli* capable of expressing *Escherichia coli* derived pyrG gene with *Corynebacterium ammoniagenes* (Japanese Published Unexamined Patent Application No. 50 276974/93) is exemplified.

55 In the same manner, the microorganism having the properties described in 2) is not necessarily a single microorganism and the properties can independently be owned by two or more microorganisms. By optionally combining the microorganisms, each sugar nucleotide of interest can be produced.

For example, it is possible to produce UDP-Glc from a UTP precursor such as orotic acid or the like and glucose using a microorganism which has the properties of microorganism described in 1) and at least one microorganism having the properties described in 2)-(i); UDP-Gal from a UTP precursor such as orotic acid or the like and galactose using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(ii); UDP-GlcNAc from a UTP precursor such as orotic acid or the like and glucosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(iii); UDP-GalNAc from a UTP precursor such as orotic acid or the like and N-acetylglucosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(iv); UDP-GlcUA from a UTP precursor such as orotic acid or the like and N-acetylglucosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(v); GDP-Man from a GTP precursor such as GMP or the like and mannose using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(vi); GDP-Fuc from a GTP precursor such as GMP or the like and mannose using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(vii); and CMP-NeuAc from a CTP precursor such as orotic acid or the like and N-acetylglucosamine or N-acetylmannosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(viii).

or N-acetylglucosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(iii); UDP-GalNAc from a UTP precursor such as orotic acid or the like and glucosamine or N-acetylglucosamine using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(iv); UDP-GlcUA from a UTP precursor such as orotic acid or the like and glucose using a microorganism which has the properties of microorganisms described in 1) and at least one microorganism having the properties described in 2)-(v); GDP-Man from a GTP precursor such as GMP or the like and mannose using a microorganism which has the properties described in 1) and at least one microorganism having the properties described in 2)-(vi); GDP-Fuc from a GTP precursor such as GMP or the like and mannose using a microorganism which has the properties described in 1) and at least one microorganism having the properties described in 2)-(vii); and CMP-NeuAc from a CTP precursor such as orotic acid or the like and N-acetylglucosamine or N-acetylmannosamine using a microorganism which has the properties described in 1) and at least one microorganism having the properties described in 2)-(viii).

As described in the foregoing, recombinant microorganisms can be used in the production of sugar nucleotides, and the genes shown in Table 2 related to the production of sugar nucleotides have been cloned from the chromosome of *Escherichia coli* and their complete nucleotide sequences have been determined.

Table 2

Genes	References
galU gene	<i>J. Biochem.</i> , <u>115</u> , 965 (1994)
ppa gene	<i>J. Bacteriol.</i> , <u>170</u> , 5901 (1988)
galK gene	<i>Nucleic Acids Res.</i> , <u>13</u> , 1841 (1985)
galT gene	<i>Nucleic Acids Res.</i> , <u>14</u> , 7705 (1986)
glmU gene	<i>J. Bacteriol.</i> , <u>175</u> , 6150 (1993)
pgm gene	<i>J. Bacteriol.</i> , <u>176</u> , 5847 (1994)
pfkB gene	<i>Gene</i> , <u>28</u> , 337 (1984)
glmM gene	<i>J. Biol. Chem.</i> , <u>271</u> , 32 (1996)
glk gene	<i>J. Bacteriol.</i> , <u>179</u> , 1298 (1997)
manB gene	<i>J. Bacteriol.</i> , <u>178</u> , 4885 (1996)
manC gene	<i>J. Bacteriol.</i> , <u>178</u> , 4885 (1996)
gmd gene	<i>J. Bacteriol.</i> , <u>178</u> , 4885 (1996)
wcaG gene	<i>J. Bacteriol.</i> , <u>178</u> , 4885 (1996)
neuA gene	<i>J. Biol. Chem.</i> , <u>264</u> , 14769 (1989)
neuB gene	<i>J. Bacteriol.</i> , <u>177</u> , 312 (1995)
nanA gene	<i>Nucleic Acids Res.</i> , <u>13</u> , 8843 (1985)
pyrG gene	<i>J. Biol. Chem.</i> , <u>261</u> , 5568 (1986)
ugd gene	<i>J. Bacteriol.</i> , <u>177</u> , 4562 (1995)

Various procedures related to recombinant DNA techniques, such as isolation and purification of plasmid DNA from *Escherichia coli* having a plasmid which contains the genes, cleavage of the plasmid DNA with restriction enzymes, isolation and purification of the cleaved DNA fragments, enzymatic linking of the DNA fragments and transformation of *Escherichia coli* using recombinant DNA, can be carried out in accordance with known processes (for example, J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, second edition, Cold Spring Harbor Laboratory (1989)). In addition, the polymerase chain reaction (referred to as "PCR" hereinafter) can be carried out, for example, using Thermal Cycler manufactured by Perkin-Elmer-Cetus.

Expression of a gene related to the production of a sugar nucleotide in a host can be effected by a procedure in which a DNA fragment containing the gene is obtained as an appropriate length of DNA fragment containing the gene using restriction enzymes or PCR and then the thus formed DNA fragment is inserted into downstream of the promoter of an expression vector, and the DNA-inserted expression vector is introduced into a host cell which is suited for the

expression vector.

Every microorganism can be used as the host, so long as it can express the gene of interest. Examples include microorganisms belonging to the genus *Escherichia*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Pseudomonas*, *Bacillus* and the like, as well as yeasts belonging to the genus *Saccharomyces*, *Candida* and the like.

With regard to the expression vector to be used, those having an ability to replicate autonomously in the above-described host or to be integrated into chromosome, and containing a promoter at the position at which transcription of the gene related to the production of a sugar nucleotide can be effected, may be used.

When the above-mentioned microorganism is used as the host, it is preferred that the expression vector of a gene related to the production of a sugar nucleotide can be replicated autonomously in the microorganism and that, at the same time, the expression vector comprises a promoter, a ribosome-binding sequence, the gene related to the production of sugar nucleotide and a transcription terminator sequence. It may also contain a regulatory gene of the promoter.

Examples of the expression vector include pBTrp2, pBTac1, pBTac2 (all manufactured by Boehringer Mannheim Co.), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 (Agric. Biol. Chem., 48, 669 (1984)), pLSA1 (Agric. Biol. Chem., 53, 277 (1989)), pGEL1 (Proc. Natl. Acad. Sci. USA., 82, 4306 (1985)), pBlue-script II SK+ (manufactured by STRATAGENE), pTrS30 (prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)), pTrS32 (prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)), pUC19 (Gene, 33, 103 (1985)), pSTV28 (manufactured by Takara Shuzo Co., Ltd.), pPA1 (Japanese Published Unexamined Patent Application No. 233798/88), pCG11 (Japanese Examined Patent Application No. 91827/94), and the like.

Any promoter can be used, so long as it can be expressed in the above-mentioned host. Examples include promoters derived from *Escherichia coli*, phage and the like, such as *trp* promoter, *lac* promoter,  $P_L$  promoter,  $P_R$  promoter, and the like. Also usable are artificially designed and modified promoters such as *trp* tandem promoter in which two *trp* promoters are connected in series, *tac* promoter, and the like.

With regard to the ribosome-binding sequence, any sequence capable of being expressed in the above-mentioned host can be used, but it is preferred to use a plasmid in which the region between a ribosome-binding sequence and an initiation codon is adjusted to a suitable distance (for example, 6 to 18 bases).

Although the transcription terminator sequence is not always necessary for the expression of genes related to the production of sugar nucleotide, it is preferred to arrange the transcription termination sequence preferably at a downstream position of the structural gene.

Any microorganism can be used as the host, so long as it can express the recombinant DNA and can apply to the sugar nucleotide formation reaction. Examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* KY8415, *Escherichia coli* NM522, *Bacillus subtilis*, *Bacillus brevis*, *Bacillus amyloliquefaciens*, *Brevibacterium* *immariorophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Brevibacterium* *flavum* ATCC 14067, *Brevibacterium* *lactofermentum* ATCC 13869, *Corynbacterium ammoniagenes* ATCC 21170, *Corynebacterium* *glutamicus* ATCC 13032, *Corynbacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas putida*, *Serratia marcescens*, and the like.

When a yeast strain is used as the host, the expression vector may be YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419) or the like.

With regard to the promoter, any promoter capable of being expressed in the yeast strain host can be used. Examples include promoters in genes of the glycolytic pathway, such as hexokinase as well as other promoters such as gal 1 promoter, gal 10 promoter, heat shock protein promoter, MFα1 promoter and CUP 1 promoter.

With regard to the host, any yeast capable of expressing recombinant DNA and applying to the sugar nucleotide formation reaction can be used. Examples include *Saccharomyces cerevisiae*, *Candida utilis*, *Candida parapsilosis*, *Candida krusei*, *Candida versatilis*, *Candida lipolytica*, *Candida zeylandioides*, *Candida guilliermondii*, *Candida albicans*, *Candida humicola*, *Pichia farinosa*, *Pichia ohmeri*, *Torulopsis candida*, *Torulopsis sphaerica*, *Torulopsis xylinus*, *Torulopsis famata*, *Torulopsis versatilis*, *Debaryomyces subglobosus*, *Debaryomyces cantarellii*, *Debaryomyces globosus*, *Debaryomyces hansenii*, *Debaryomyces japonicus*, *Zygosaccharomyces rouxii*, *Zygozacharomyces baillii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Hansenula anomala*, *Hansenula jadinii*, *Brettanomyces lambicus*, *Brettanomyces anomalus*, *Schizosaccharomyces pombe*, *Trichosporon pullulans*, *Schwanniomyces alluvius*, and the like.

Culturing of the microorganism for use in the present invention can be carried out in accordance with the usual culturing process. The medium for use in the culturing of the microorganism may be either a nutrient medium or a synthetic medium, so long as it contains carbon sources, nitrogen sources, inorganic salts and the like which can be assimilated by the microorganism and it can perform culturing of the microorganism efficiently.

Examples of the carbon sources include those which can be assimilated by each microorganism, such as carbohydrates (for example, glucose, fructose, sucrose, lactose, maltose, mannitol, sorbitol, molasses, starch, starch hydrolysate, etc.), organic acids (for example, pyruvic acid, lactic acid, citric acid, fumaric acid, etc.), various amino acids (for example, glutamic acid, methionine, lysine, etc.), and alcohols (for example, ethanol, propanol, glycerol, etc.). Also use-

ful are natural organic nutrient sources, such as rice bran, cassava, bagasse, corn steep liquor, and the like.

Examples of the nitrogen sources include various inorganic and organic ammonium salts (for example, ammonia, ammonium chloride, ammonium sulfate, ammonium carbonate, ammonium acetate, ammonium phosphate, etc.), amino acids (for example, glutamic acid, glutamine, methionine, etc.), peptone, NZ amine, corn steep liquor, meat extract, yeast extract, malt extract, casein hydrolysate, soybean meal, fish meal or a hydrolysate thereof and the like.

5 Examples of the inorganic substances include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, magnesium phosphate, magnesium sulfate, magnesium chloride, sodium chloride, calcium chloride, ferrous sulfate, manganese sulfate, copper sulfate, zinc sulfate, calcium carbonate, and the like. Vitamins, amino acids, nucleic acids and the like may be added as occasion demands.

10 The culturing is carried out under aerobic conditions by shaking culture, aeration stirring culture or the like means. The culturing temperature is preferably from 15 to 45°C, and the culturing time is generally from 5 to 96 hours. The pH of the medium is maintained at 3.0 to 9.0 during the culturing. Adjustment of the medium pH is carried out using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia and the like. Also, antibiotics (for example, ampicillin, tetracycline, etc.) may be added to the medium during the culturing as occasion demands.

15 When a microorganism transformed with an expression vector in which an inducible promoter is used as the promoter is cultured, an inducer may be added to the medium as occasion demands. For example, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) or the like may be added to the medium when a microorganism transformed with an expression vector containing lac promoter is cultured, or indoleacrylic acid (IAA) or the like may be added thereto when a microorganism transformed with an expression vector containing try promoter is cultured.

20 When two or more microorganisms are used in the sugar nucleotide production of the present invention, the microorganisms may be separately cultured to use the resulting culture broths in the sugar nucleotide production or inoculated simultaneously into a single culture vessel to carry out mixed culturing and to use the resulting culture broth in the sugar nucleotide production. In an alternative way, one of the microorganisms is firstly cultured, the remaining microorganism is inoculated during or after the culturing and cultured, and then the resulting culture broth is used in the sugar nucleotide production. In another alternative way, a microorganism having the properties described in 1) and a microorganism having the properties described in 2) may be separately cultured and used in the sugar nucleotide production using the resulting culture broths.

25 The microbial culture broth obtained by the culturing or a treated product of the culture broth obtained by treating the culture broth in various ways can be used as an enzyme source for the formation of a sugar nucleotide in an aqueous medium.

30 Examples of the treated product of the culture broth include a concentrated product of the culture broth, a dried product of the culture broth, a culture supernatant obtained by centrifuging the culture broth, a concentrated product of the culture supernatant, an enzyme preparation obtained from the culture supernatant, cells (including microbial cells) obtained by centrifuging the culture broth, a dried product of the cells, a freeze-dried product of the cells, a surfactant-treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells, an immobilized product of the cells and an enzyme preparation obtained by extraction from the cells.

35 The amount of the enzyme source used in the formation of the sugar nucleotide is within the range of from 1 to 500 g/l, preferably from 5 to 300 g/l, as wet cells. When the reaction is carried out in an aqueous medium using two or more microorganisms simultaneously, amount of the total wet cells of the microorganisms in the aqueous medium is within the range of from 2 to 500 g/l, preferably from 5 to 400 g/l.

40 Examples of the aqueous medium used in the formation of the sugar nucleotide include water, buffer solutions (for example, those of phosphate, carbonate, acetate, borate, citrate, Tris, etc.), alcohols (for example, methanol, ethanol, etc.), esters (for example, ethyl acetate, etc.), ketones (for example, acetone, etc.), amides (for example, acetamide, etc.), and the like. The microbial culture broth used as the enzyme source may also be used as the aqueous medium.

45 Examples of the nucleotide precursor used in the formation of the sugar nucleotide include orotic acid, uracil, orotidine, uridine, cytosine, cytidine, adenine, adenosine, guanine, guanosine, hypoxanthine, inosine, xanthine, xanthosine, inosine-5'-monophosphate, xanthosine-5'-monophosphate, guanosine-5'-monophosphate, uridine-5'-monophosphate, cytidine-5'-monophosphate, and the like. Preferred are orotic acid and guanosine-5'-monophosphate. The nucleotide precursor may be in the form of a purified product or in the form of a salt of the precursor, and a culture broth containing the precursor produced by the fermentation of a microorganism or the precursor roughly purified from the culture broth may also be used as the nucleotide precursor, so long as its impurities do not inhibit the reaction. The nucleotide precursor is used at a concentration of from 0.1 mM to 1.0 M, preferably from 0.01 to 0.3 M.

50 Examples of the sugar used in the formation of the sugar nucleotide include glucose, fructose, galactose, glucose-6-phosphate, N-acetylglucosamine, N-acetylgalactosamine, mannose, fucose, N-acetylmannosamine, acetylneurameric acid and the like, and derivatives thereof. The sugar may be either in the form of a purified product or in the form of a material containing the same, so long as impurities in the material do not inhibit the reaction. The sugar is used at a

concentration of from 0.1 mM to 2.0 M, by adding it in one portion when the reaction is started or in portions or continuously during the reaction.

In the formation of the sugar nucleotide, an energy source necessary for the regeneration of ATP, a coenzyme, a phosphate ion, a magnesium ion, a chelating agent (for example, phytic acid, etc.), a surfactant and an organic solvent may be added as occasion demands.

Examples of the energy source include carbohydrate (for example, glucose, fructose, sucrose, lactose, maltose, mannitol, sorbitol, etc.), organic acids (for example, pyruvic acid, lactic acid, acetic acid, etc.), amino acids (for example, glycine, alanine, aspartic acid, glutamic acid etc.), molasses, starch hydrolysate, and the like, which may be used at a concentration of from 1.0 mM to 2.0 M.

Examples of the phosphate ion include orthophosphoric acid, polyphosphoric acids (for example, pyrophosphoric acid, tripolyphosphoric acid, tetrapolyphosphoric acid, tetrapolymetaphosphoric acid, etc.), polymetaphosphoric acids, inorganic phosphates (for example, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, etc.), and the like, which may be used at a concentration of from 1.0 mM to 1.0 M.

Examples of the magnesium ion include inorganic magnesium salts (for example, magnesium sulfate, magnesium nitrate, magnesium chloride, etc.), organic magnesium salts (for example, magnesium citrate, etc.), and the like, which may be used at a concentration of generally from 1 to 100 mM.

Examples of the surfactant include those which can enhance the production of various sugar nucleotides, such as nonionic surfactants (for example, polyoxyethylene octadecylamine (e.g., Nymeen S-215, manufactured by Nippon Oils and Fats Co., etc.), cationic surfactants (for example, cetyl trimethylammonium bromide, alkyldimethyl benzylammonium chloride (e.g., Cation F2-40E, manufactured by Nippon Oils and Fats Co.) etc.), anionic surfactants (for example, lauroyl sarcosinate, etc.) and tertiary amines (for example, alkyldimethylamine (e.g., Tertiary Amine FB, manufactured by Nippon Oils and Fats Co., etc.), which may be used alone or as a mixture of two or more. The surfactant may be used at a concentration of generally from 0.1 to 50 g/L.

Examples of the organic solvent include xylene, toluene, aliphatic alcohol, acetone, ethyl acetate, and the like, which may be used at a concentration of generally from 0.1 to 50 mL.

The reaction for forming a sugar nucleotide can be carried out in an aqueous medium at pH of from 5 to 10, preferably from 6 to 9, at a temperature of from 20 to 50°C and for a period of from 2 to 96 hours.

The sugar nucleotide can be formed by the process. Examples include a uridine diphosphate compound, a guanosine diphosphate compound, a cytidine monophosphate compound and the like. Specific examples include sugar nucleotides selected from UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-GlcUA, GDP-Man, GDP-Fuc, CMP-NeuAc, and the like, and derivatives thereof.

Determination of the sugar nucleotide formed in the aqueous medium can be carried out in accordance with a known method, for example, isolation and determination of UDP-Glc and UDP-Gal can be carried out by high performance liquid chromatography (referred to as "HPLC" hereinafter) method described in *Anal. Biochem.*, 216, 188 (1994). In addition, isolation and determination of UDP-GlcNAc, GDP-Man, GDP-Fuc and CMP-NeuAc can be carried out by HPLC under the following conditions:

Elution solution:  
40 0.1 M KH<sub>2</sub>PO<sub>4</sub> (adjust to pH 3.2 with H<sub>3</sub>PO<sub>4</sub>)

Flow rate:  
1 mL/min

Column:  
45 Partisil-10 SAX (manufactured by Whatman)

Detection:  
UV 262 nm

Determination:  
Calculated by comparing standard absorbance values

50 Recovery of the sugar nucleotide formed in the reaction solution can be carried out in the usual way using activated carbon, an ion exchange resin and the like, for example, UDP-Gal and UDP-Glc can be recovered in accordance with the process described in *J. Org. Chem.*, 57, 152 (1992), and UDP-GlcNAc with the process described in *J. Org. Chem.*, 57, 146 (1992).

With regard to the microorganisms, animal cells or insect cells eligible for in the production of the complex carbohydrate of the present invention, all microorganisms, animal cells or insect cells capable of producing the complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor can be used. Examples of such microorganisms, animal cells or insect cells include those which have the activities of glucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyl-

transferase, sialyltransferase, fucosyltransferase, and the like.

In addition, microorganisms, animal cells or insect cells modified by recombinant DNA techniques can also be used in a similar way to the case of the above-mentioned sugar nucleotide production. Examples of such microorganisms, animal cells or insect cells include *Escherichia coli* which expresses the ceramide glucosyltransferase gene derived from human melanoma cell line SK-Mel-28 (Proc. Natl. Acad. Sci. USA., 93, 4638 (1996)), human melanoma cell line WM266-4 which produces  $\beta$ 1,3-galactosyltransferase (ATCC CRL 1676), recombinant cell line such as namalwa cell line KJM-1 or the like which contains the  $\beta$ 1,3-galactosyltransferase gene derived from the human melanoma cell line WM266-4 (Japanese Published Unexamined Patent Application No. 181759/94), *Escherichia coli* (EMBO J., 9, 3171 (1990)) or *Saccharomyces cerevisiae* (Biochem. Biophys. Res. Commun., 201, 160 (1994)) which expresses the  $\beta$ 1,4-galactosyltransferase gene derived from human HeLa cells, COS-7 cell line (ATCC CRL 1651) which expresses the rat  $\beta$ 1,6-N-acetylglucosaminyltransferase gene (J. Biol. Chem., 268, 15381 (1993)), St9 cell line which expresses human N-acetylglucosaminyltransferase gene (J. Biochem., 118, 568 (1995)), *Escherichia coli* which expresses human glucuronosyltransferase (Biochem. Biophys. Res. Commun., 196, 473 (1993)), namalwa cell line which expresses human  $\alpha$ 1,3-fucosyltransferase (J. Biol. Chem., 269, 14730 (1994)), COS-1 cell line which expresses human  $\alpha$ 1,3/1,4-fucosyltransferase (Genes Dev., 4, 1288 (1990)), COS-1 cell line which expresses human  $\alpha$ 1,2-fucosyltransferase (Proc. Natl. Acad. Sci. USA., 87, 6674 (1990)), COS-7 cell line which expresses chicken  $\alpha$ 2,6-sialyltransferase (Eur. J. Biochem., 219, 375 (1994)), COS cell line which expresses human  $\alpha$ 2,8-sialyltransferase (Proc. Natl. Acad. Sci. USA., 91, 7952 (1994)), *Escherichia coli* which expresses  $\beta$ 1,3-N-acetylglucosaminyltransferase,  $\beta$ 1,4-galactosyltransferase,  $\beta$ 1,3-N-acetylgalactosaminyltransferase or  $\alpha$ 1,4-galactosyltransferase derived from *Neisseria* (WO 96/10086), *Escherichia coli* which expresses *Neisseria*-derived  $\alpha$ 2,3-sialyltransferase (J. Biol. Chem., 271, 28271 (1996)), *Escherichia coli* which expresses *Helicobacter pylori*-derived  $\alpha$ 1,3-fucosyltransferase (J. Biol. Chem., 272, 21349 and 21357 (1997)), *Escherichia coli* which expresses yeast-derived  $\alpha$ 1,2-mannosyltransferase (J. Org. Chem., 58, 3985 (1993)), and the like.

When a microorganism is used for producing the complex carbohydrate of the present invention, the microorganism can be cultured using the same medium under the same culture conditions as in the case of the above-mentioned microorganism capable of producing a sugar nucleotide from a nucleotide precursor and a sugar.

When animal cells are used for producing the complex carbohydrate of the present invention, the preferred culture medium is generally RPMI 1640 medium, Eagle's MEM medium or a medium thereof modified by further adding fetal calf serum, and the like. The culturing is carried out under certain conditions, for example, in the presence of 5% CO<sub>2</sub>. The culturing is carried out at a temperature of preferably from 20 to 40°C for a period of generally from 3 to 14 days. As occasion demands, antibiotics may be added to the medium.

30 The culturing is carried out in a culture medium. As occasion demands, antibiotics may be added to the medium.  
When insect cells are used for producing the complex carbohydrate of the present invention, culturing of the insect  
35 cells can be carried out in accordance with the known process (*J. Biol. Chem.*, **268**, 12609 (1993)).  
The culture broth of a microorganism, an animal cell line or an insect cell line obtained by the culturing and a treated  
product of the culture broth obtained by treating the culture broth in various ways can be used as an enzyme source for  
producing the complex carbohydrate in an aqueous medium.  
After the culturing, a part of the culture broth, a dried

35 forming the complex carbohydrate in an aqueous medium.

Examples of the treated product of the culture broth include a concentrated product of the culture broth, a dried product of the culture broth, a culture supernatant obtained by centrifuging the culture broth, a concentrated product of the culture supernatant, an enzyme preparation obtained from the culture supernatant, cells (including microbial cells) obtained by centrifuging the culture broth, a dried product of the cells, a freeze-dried product of the cells, a surfactant-treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells, an immobilized product of the cells and an enzyme preparation obtained by extraction from the cells.

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The enzyme source used in the formation of the complex carbohydrate is typically within the range of from 0.1 mU/l to 10,000 U/l, preferably from 1 mU/l to 1,000 U/l (where 1 unit (U) is the amount of the enzyme activity which can form a complex carbohydrate within 1 minute at 37°C).

45 to 10,000 U/l, preferably from 1 mM to 10 mM. 1 μmole of the complex carbohydrate within 1 minute at 37°C.

Examples of the aqueous medium used in the formation of the complex carbohydrate include water, buffer solutions (for example, those of phosphate, carbonate, acetate, borate, citrate, Tris, etc.), alcohols (for example, methanol, ethanol, etc.), esters (for example, ethyl acetate, etc.), ketones (for example, acetone, etc.), amides (for example, acetamide, etc.), and the like. Each of the culture broths of microorganisms, animal cells or insect cells used as the enzyme sources may also be used as the aqueous medium.

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As occasion demands, chelating agents (for example, phytic acid, etc.), inorganic salts (for example,  $MnCl_2$ , etc.), or acetone-ethanol and the like may be added.

As the sugar nucleotide used in the formation of the complex carbohydrate, the above-mentioned reaction solution obtained by the sugar nucleotide formation or the sugar nucleotide purified from the reaction solution can be used at a concentration of from 0.01 mM to 2.0 M.

In addition, a sugar nucleotide can be supplied in the complex carbohydrate formation reaction solution by forming a complex with the above-mentioned process.

With regard to the complex carbohydrate precursor used in the formation of the complex carbohydrate, any material can be used, so long as it can be used as the substrate of glycosyltransferase. Examples include monosaccharides, oligosaccharides, monosaccharides or oligosaccharides linked to a carrier or the like, proteins, peptides, lipids, glycoproteins, glycolipids, glycopeptides, steroid compounds, and the like.

Specific examples include glucose, galactose, mannose, sialic acid, N-acetylglucosamine, N-acetylgalactosamine, lactose, N-acetyllactosamine, lacto-N-biose, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc, GlcNAc $\beta$ 1-4Gal $\beta$ 1-4Glc, globotriose, Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 6'-sialyl-N-acetyllactosamine, 6'-sialyl-N-acetyllactosamine, sialylacto-N-biose, Lewis X, Lewis a, lacto-N-tetraose, lactodifucotetraose, 3-sialyl-3-fucosyllactose, sialyl-Lewis X, sialyl-Lewis a, lacto-N-fucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, LS-tetrasaccharide a, LS-tetrasaccharide b, LS-tetrasaccharide c, lacto-N-fucopentaose and derivatives thereof, serine, threonine, asparagine and peptides containing these ( $\alpha$ 2,3) sialyllacto-N-neotetraose and derivatives thereof, ceramide and derivatives thereof, and the like. The complex carbohydrate precursor can be used at a concentration of from 0.01 mM to 2.0 M.

Examples of the complex carbohydrate of the present invention include complex carbohydrates containing at least one sugar selected from glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid, mannose, N-acetylmannosamine, fucose, sialic acid, lactose, N-acetyllactosamine, lacto-N-biose, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc, GlcNAc $\beta$ 1-4Gal $\beta$ 1-4Glc, globotriose, Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-N-acetyllactosamine, sialylacto-N-biose, Lewis X, Lewis a, lacto-N-tetraose, lactodifucotetraose, 3'-sialyl-3-fucosyllactose, sialyl-Lewis X, sialyl-Lewis a, lacto-N-fucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, LS-tetrasaccharide a, LS-tetrasaccharide b, LS-tetrasaccharide c, ( $\alpha$ 2,3)sialyllacto-N-neotetraose, lacto-N-difucohexaose I, lacto-N-difucohexaose II, lacto-N-hexaose, lacto-N-neohexaose, disialyllacto-N-tetraose and derivatives thereof, and complex carbohydrates which contain the just described complex carbohydrates. Specifically, they include complex carbohydrates which contain a sugar having a bond selected from Gal $\beta$ 1-3Glc, Gal $\beta$ 1-4Glc, Gal $\beta$ 1-3GlcNAc, Gal $\beta$ 1-4GlcNAc, Gal $\beta$ 1-3Gal, Gal $\beta$ 1-4Gal, Gal $\beta$ 1-3GalNAc, Gal $\beta$ 1-4GalNAc, Gal $\alpha$ 1-3Glc, Gal $\alpha$ 1-4Glc, Gal $\alpha$ 1-3GlcNAc, Gal $\alpha$ 1-4GlcNAc, Gal $\alpha$ 1-3Gal, Gal $\alpha$ 1-4Gal, Gal $\alpha$ 1-3GalNAc, Gal $\alpha$ 1-4GalNAc, GlcNAc $\beta$ 1-3Gal, GlcNAc $\beta$ 1-4Gal, GlcNAc $\beta$ 1-6Gal, GlcNAc $\beta$ 1-3Glc, GlcNAc $\beta$ 1-4Glc, GlcNAc $\beta$ 1-3GlcNAc, GlcNAc $\beta$ 1-4GlcNAc, GlcNAc $\beta$ 1-6GalNAc, GlcNAc $\beta$ 1-2Man, GlcNAc $\beta$ 1-3GlcNAc, GalNAc $\beta$ 1-3Gal, GalNAc $\beta$ 1-4Gal, GalNAc $\beta$ 1-4GlcNAc, GalNAc $\alpha$ 1-3GalNAc, Man $\beta$ 1-4Man, GlcNAc $\beta$ 1-6Man, GalNAc $\beta$ 1-3Gal, GalNAc $\beta$ 1-4GlcNAc, GalNAc $\alpha$ 1-3GlcNAc, Man $\alpha$ 1-6Man, Man $\alpha$ 1-3Man, Man $\alpha$ 1-2Man, GlcUA $\beta$ 1-4GlcN, GlcUA $\beta$ 1-3Gal, GlcUA $\beta$ 1-3GlcNAc, GlcUA $\beta$ 1-4GlcNAc, NeuAca $\alpha$ 2-3Gal, NeuAca $\alpha$ 2-6Gal, NeuAca $\alpha$ 2-3GlcNAc, NeuAca $\alpha$ 2-6GlcNAc, NeuAca $\alpha$ 2-3GlcNAc, NeuAca $\alpha$ 2-6Gal and Fuc $\alpha$ 1-6GlcNAc, NeuAca $\alpha$ 2-8NeuAc, Fuc $\alpha$ 1-3Glc, Fuc $\alpha$ 1-4Glc, Fuc $\alpha$ 1-3GlcNAc, Fuc $\alpha$ 1-4GlcNAc, Fuc $\alpha$ 1-2Gal and Fuc $\alpha$ 1-6GlcNAc; and complex carbohydrates which contain the just described complex carbohydrates. In this case, the number of sugars contained in the complex carbohydrate having the sugars may be 10 or below, or 6 or below.

As specific processes for producing the complex carbohydrates,

(1) lactose can be formed from orotic acid, galactose and glucose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(2) N-acetyllactosamine can be formed from orotic acid, galactose and N-acetylglucosamine by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(3) 3'-sialyllactose can be formed from orotic acid, N-acetylmannosamine, pyruvic acid and lactose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha$ 2,3-sialyltransferase (*J. Biol. Chem.*, 271, 28271 (1996)), a microorganism having the ability to produce CTP from a precursor of CTP and a microorganism having the ability to produce CMP-NeuAc from a sugar and CTP, or treated products of these culture broths, as enzyme sources,

(4) 3'-sialyl-N-acetyllactosamine can be formed from orotic acid, N-acetylmannosamine, pyruvic acid and N-acetyl-lactosamine by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha$ 2,3-sialyltransferase (*J. Biol. Chem.*, 271, 28271 (1996)), a microorganism having the ability to produce CTP from a precursor of CTP and a microorganism having the ability to produce CMP-NeuAc from a sugar and CTP, or treated products of these culture broths, as enzyme sources,

(5) 6'-sialyl-N-acetyllactosamine can be formed from orotic acid, N-acetylmannosamine, pyruvic acid and N-acetyl-lactosamine by carrying out an enzyme reaction using culture broths of COS-7 cell line having the ability to express chicken-derived  $\alpha$ 2,6-sialyltransferase (*Eur. J. Biochem.*, 219, 375 (1994)), a microorganism having the ability to

produce CTP from a precursor of CTP and a microorganism having the ability to produce CMP-NeuAc from a sugar and CTP, or treated products of these culture broths, as enzyme sources,

(6) GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc can be formed from orotic acid, N-acetylglucosamine and lactose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,3-N-acetylglucosaminyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-GlcNAc from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(7) lacto-N-tetraose can be formed from orotic acid, galactose and GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc by carrying out an enzyme reaction using culture broths of human melanoma cell line WM266-4 having the ability to produce  $\beta$ 1,3-galactosyltransferase (ATCC CRL 1676) or a transformant such as of namalwa cell line KJM-1 having the ability to express  $\beta$ 1,3-galactosyltransferase gene derived from human melanoma cell line WM266-4 (Japanese Published Unexamined Patent Application No. 181759/94), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(8) lacto-N-neotetraose can be formed from orotic acid, galactose and GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc by carrying out an enzyme reaction using culture broths of *Escherichia coli* (EMBO J., 9, 3171 (1990)) or *Saccharomyces cerevisiae* (*Biochem. Biophys. Res. Commun.*, 201, 160 (1994)) having the ability to express  $\beta$ 1,4-galactosyltransferase gene derived from human HeLa cell line, a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(9) lacto-N-neotetraose can be formed from orotic acid, galactose and GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(10) lacto-N-neotetraose can be formed from orotic acid, N-acetylglucosamine, galactose and lactose by carrying out an enzyme reaction using culture broths of a microorganism which can express *Neisseria*-derived  $\beta$ 1,3-N-acetylglucosaminyltransferase (WO 96/10086), a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP, a microorganism having the ability to produce UDP-GlcNAc from a sugar and UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(11) ( $\alpha$ 2,3)sialyllacto-N-neotetraose can be formed from orotic acid, N-acetylmannosamine, pyruvic acid and lacto-N-neotetraose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha$ 2,3-sialyltransferase (*J. Biol. Chem.*, 271, 28271 (1996)), a microorganism having the ability to produce CTP from a precursor of CTP and a microorganism having the ability to produce CMP-NeuAc from a sugar and CTP, or treated products of these culture broths, as enzyme sources,

(12) lacto-N-fucopentaose III can be formed from GMP, mannose and lacto-N-neotetraose by carrying out an enzyme reaction using culture broths of namalwa cell line having the ability to express human-derived  $\alpha$ 1,3-fucosyltransferase (*J. Biol. Chem.*, 269, 14730 (1994)), a microorganism having the ability to produce GTP from a precursor of GTP and a microorganism having the ability to produce GDP-Fuc from a sugar and GTP, or treated products of these culture broths, as enzyme sources,

(13) lacto-N-fucopentaose III can be formed from GMP, mannose and lacto-N-neotetraose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Helicobacter pylori*-derived  $\alpha$ 1,3-fucosyltransferase (*J. Biol. Chem.*, 272, 21349 and 21357 (1997)), a microorganism having the ability to produce GTP from a precursor of GTP and a microorganism having the ability to produce GDP-Fuc from a sugar and GTP, or treated products of these culture broths, as enzyme sources,

(14) globotriose can be formed from orotic acid, galactose and lactose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(15) globotriose can be formed from orotic acid, galactose and glucose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\beta$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to express *Neisseria*-derived  $\alpha$ 1,4-galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources,

(16)  $\text{Gal}\alpha 1\text{-}4\text{Gal}\beta 1\text{-}4\text{GlcNAc}$  can be formed from orotic acid, galactose and N-acetyllactosamine by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha 1\text{-}4$ -galactosyltransferase (WO 96/10086), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources.

(17) lacto-N-biose can be formed from orotic acid, galactose and N-acetylglucosamine by carrying out an enzyme reaction using culture broths of an animal cell line having the ability to express human-derived  $\beta 1\text{-}3$ -galactosyltransferase (Japanese Published Unexamined Patent Application No. 181759/94), a microorganism having the ability to produce UTP from a precursor of UTP and a microorganism having the ability to produce UDP-Gal from a sugar and UTP, or treated products of these culture broths, as enzyme sources.

(18) sialylacto-N-biose can be formed from orotic acid, N-acetylmannosamine, pyruvic acid and lacto-N-biose by carrying out an enzyme reaction using culture broths of a microorganism having the ability to express *Neisseria*-derived  $\alpha 2\text{-}3$ -sialyltransferase (*J. Biol. Chem.*, **271**, 28271 (1996)), a microorganism having the ability to produce CTP from a precursor of CTP and a microorganism having the ability to produce CMP-NeuAc from a sugar and CTP, or treated products of these culture broths, as enzyme sources.

(19) sialyl-Lewis X can be formed from GMP, mannose and 3'-sialyl-N-acetyllactosamine by carrying out an enzyme reaction using culture broths of an animal cell line having the ability to express human-derived  $\alpha 1\text{-}3$ -fucosyltransferase (*J. Biol. Chem.*, **269**, 14730 (1994)), a microorganism having the ability to produce GTP from a precursor of GTP and a microorganism having the ability to produce GDP-Fuc from a sugar and GTP, or treated products of these culture broths, as enzyme sources.

(20) sialyl-Lewis X can be formed from GMP, mannose and sialylacto-N-biose by carrying out an enzyme reaction using human  $\alpha 1\text{-}3/1\text{-}4$ -fucosyltransferase (*Carbohydrate Research*, **190**, 1 (1989)) and culture broths of a microorganism having the ability to produce GTP from a precursor of GTP and a microorganism having the ability to produce GDP-Fuc from a sugar and GTP, or treated products of these culture broths, as enzyme sources, and

(21) Man $\alpha 1\text{-}2$ Man can be formed from GMP and mannose by carrying out an enzyme reaction using culture broths of *Escherichia coli* having the ability to express yeast-derived  $\alpha 1\text{-}2$ -mannosyltransferase (*J. Org. Chem.*, **58**, 3985 (1993)), a microorganism having the ability to produce GTP from a precursor of GTP and a microorganism having the ability to produce GDP-Man from a sugar and GTP, or treated products of these culture broths, as enzyme sources.

Processes for producing complex carbohydrates are not limited to the above-mentioned examples, and any other sugar chain can be produced industrially using a nucleotide precursor, a sugar and a complex carbohydrate precursor as the sole starting materials, within the range of glycosyltransferases which can be combined with the sugar nucleotide production process described herein and of the substrate specificity acceptable by the enzymes.

Examples of the complex carbohydrate to be produced by the production process of the present invention include

(1) complex carbohydrates involved in the infection with pathogenic microorganisms and viruses, such as complex carbohydrates which are recognized as receptors of pathogenic microorganisms and viruses.

(2) complex carbohydrates which are recognized as receptors of toxins produced by pathogenic microorganisms and viruses.

(3) complex carbohydrates which are concerned, for example, in cell adhesion, recognition of foreign substances and binding of various types of lymphokine in the living body, and complex carbohydrates which contain one or a plurality of sugars such as glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid, mannose, N-acetylmannosamine, fucose, sialic acid and the like, in a chemically acceptable binding mode. More specific examples include

(1) complex carbohydrates which are contained in the milk of human and animals, and involved in the protection of infants from microbial infection, for example, complex carbohydrates, such as lacto-N-tetraose, lacto-N-neotetraose, and the like.

(2) receptor complex carbohydrates which recognize microorganisms, such as *Escherichia coli*, *Propionibacterium granulosum*, *Mycobacterium tuberculosis*, *Moraxella catarrhalis*, *Candida albicans*, *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Actinomyces naeslundii*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Haemophilus influenzae*, and the like.

(3) receptor complex carbohydrates of viruses, such as influenza virus, coronavirus, Sendai virus, newcastle disease virus, reovirus, rotavirus, AIDS (HIV) virus, and the like.

(4) receptor complex carbohydrates of protozoa, such as *Cryptosporidium*, *Trypanosoma*, and the like.

(5) receptor complex carbohydrates having the affinity for toxins, such as cholera toxin, *Escherichia coli* heat-labile toxin, botulinum toxin, clostridial  $\delta$  toxin, clostridial A toxin, Shiga toxin, Vero toxin, Shiga toxin-like toxin,

*Vibrio parahaemolyticus* heat-stable toxin, tetanus toxin, and the like,  
 (6) cancer-related complex carbohydrates such as gangliosides (for example, GD3, GM3, etc.), globoside glycolipids, and the like,  
 5 (7) complex carbohydrates which are concerned in the adhesion of leukocytes to inflammatory regions and modification of their functions, such as sialyl-Lewis X sugar chain, and the like,  
 (8) complex carbohydrates concerned in autoimmune diseases, such as rheumatoid arthritis, IgA glomerulonephritis, and the like, and  
 (9) complex carbohydrates which are recognized by various lectin-like substances concerned in the recognition of foreign bodies and cancer cells.

10 Determination of the complex carbohydrate formed in the aqueous medium can be carried out in accordance with known methods (*Proc. Natl. Acad. Sci. USA.*, 85, 3289 (1988), *Anal. Biochem.*, 174, 459 (1988)).

Recovery of the complex carbohydrate formed in the reaction solution can be carried out in the usual way using activated carbon, an ion exchange resin and the like, for example, N-acetyllactosamine can be recovered in accordance with the process described in *J. Org. Chem.*, 47, 5416 (1982).

15 Examples of the present invention are given below by way of illustration and not by way of limitation.

#### BEST MODE OF CARRYING OUT THE INVENTION

20 Example 1: Construction of recombinant plasmid capable of expressing galU and ppa

Construction process of recombinant plasmid pNT12 capable of expressing galU and ppa is described in the following (Figs. 1 and 2).

25 1) Construction of expression vector containing  $P_L$  promoter

Construction of pPA31 and pPAC31 as  $P_L$  promoter-containing expression vectors were carried out in the following manner (Fig. 1).

30 *Escherichia coli* JM109/pTrS30 (FERM BP-5407) which has tryptophan promoter-containing plasmid pTrS30 and another *Escherichia coli* which has  $P_L$  promoter-containing plasmid pPA1 (Japanese Published Unexamined Patent Application No. 233798/88) and  $P_L$  promoter- and cl857 repressor-containing plasmid pPAC1 (FERM BP-6054) were separately inoculated into LB medium (10 g/l Bacto-Tryptone (manufactured by Difco), 5 g/l Yeast Extract (manufactured by Difco) and 5 g/l NaCl, adjusted to pH 7.2) and cultured at 30°C for 18 hours.

35 From the cells obtained by the culturing, pTrS30, pPA1 and pPAC1 plasmid DNAs were isolated and purified by the above-mentioned known processes.

A 0.2 µg portion of the thus purified pTrS30 DNA was cleaved with restriction enzymes *Pst*I and *Cla*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.4 kb was recovered using Gene Clean II Kit (manufactured by Bio101). A 0.5 µg portion of the purified pPA1 DNA was cleaved with restriction enzymes *Pst*I and *Cla*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.0 kb was recovered in the same manner.

40 Using a ligation kit (TAKARA Ligation Kit, manufactured by Takara Shuzo Co., Ltd.), the fragments of 3.4 kb and 1.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 37°C.

45 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, the  $P_L$  promoter-effected expression vector pPA31 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 1).

A 0.2 µg portion of the purified pPA31 DNA was cleaved with restriction enzymes *Pst*I and *Cla*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.4 kb was recovered using Gene Clean II Kit. A 0.5 µg portion of the purified pPAC1 DNA was cleaved with restriction enzymes *Pst*I and *Cla*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 2.3 kb was recovered in the same manner.

50 Using a ligation kit, the fragments of 3.4 kb and 2.3 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 37°C.

55 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned

known process, the  $P_L$  promoter-effected cl857 repressor-containing expression vector pPAC31 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 1).

2) Construction of galU expression plasmid

Chromosomal DNA of *Escherichia coli* W3110 was isolated and purified by a known process (for example, Current Protocols in Molecular Biology, John Wiley and Sons Inc. (1994)).

The sense strand DNA primer shown in SEQ ID NO:1 and the antisense strand DNA primer shown in SEQ ID NO:2 were synthesized using 380A DNA Synthesizer manufactured by Applied Biosystems.

The PCR process was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template. The PCR was effected using 40  $\mu$ l of a reaction solution containing 0.04  $\mu$ g of the W3110 chromosomal DNA, 0.5  $\mu$ M of each primer, 1.0 unit of TAKARA Ex Taq (manufactured by Takara Shuzo Co., Ltd.), 4  $\mu$ l of 10  $\times$  Ex Taq buffer (manufactured by Takara Shuzo Co., Ltd.) and 200  $\mu$ M of each deoxNTP, and repeating 30 cycles of the reaction, each cycle containing 94°C for 1 minute, 42°C for 2 minutes and 72°C for 3 minutes.

A 1/10 volume of the reaction solution was subjected to agarose gel electrophoresis to verify amplification of the fragment of interest, and then the remaining reaction solution was mixed with the same volume of phenol/chloroform (1 vol/1 vol) solution with saturated TE (10 mM Tris-HCl buffer (pH 8.0) and 1 mM EDTA). The mixture solution was centrifuged, and the thus obtained upper layer was mixed with 2 volumes of cold ethanol and allowed to stand for 30 minutes at -80°C. The solution after standing was centrifuged to obtain a precipitate of DNA. The precipitate was washed with 70% cold ethanol and dried *in vacuo* to recover the precipitate. Hereinafter, the steps starting from the addition of phenol/chloroform solution with saturated TE until the recovery of the ethanol-washed DNA are referred to as an ethanol precipitation process.

The DNA precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 0.9 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pPA31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.2 kb was recovered in the same manner.

Using a ligation kit, the fragments of 0.9 kb and 4.2 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* KY8415 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, galU expression plasmid pNT9 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 2).

3) Construction of simultaneous galU, ppa expression plasmid

The sense strand DNA primer shown in SEQ ID NO:3 and the antisense strand DNA primer shown in SEQ ID NO:4 were synthesized, and the PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template, under the same conditions as described in the foregoing.

After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Bam*HI and *Sal*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.0 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pNT9 DNA obtained in Example 1-2) was cleaved with restriction enzymes *Bam*HI and *Sal*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.9 kb was recovered in the same manner.

Using a ligation kit, the fragments of 1.0 kb and 4.9 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* KY8415 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, the galU, ppa simultaneous expression plasmid pNT12 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 2).

A 0.5  $\mu$ g portion of the pNT12 DNA was cleaved with restriction enzymes *Eco*RI and *Sal*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 2.2 kb was recovered using Gene Clean II Kit. Separately from this, a 0.2  $\mu$ g portion of pSTV28 DNA (manufactured by Takara Shuzo Co., Ltd.) was cleaved with restriction enzymes *Eco*RI and *Sal*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

Using a ligation kit, the fragments of 2.2 kb and 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 10 µg/ml chloramphenicol and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in the usual way, the galU, ppa simultaneous expression plasmid pNT32 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 2).

Example 2: Production of UDP-Glc

10 *Escherichia coli* KY8415/pNT12 obtained in Example 1 was inoculated into a 1 L baffled conical flask containing 125 ml of LB medium supplemented with 50 µg/ml ampicillin and cultured at 30°C for 17 hours under shaking at 220 rpm. A 125 ml portion of the culture broth was inoculated into a 5 L culture vessel containing 2.5 L of an aqueous medium (without adjusting pH) which contains 10 g/l glucose, 12 g/l Bacto-Tryptone (manufactured by Difco), 24 g/l Yeast Extract (manufactured by Difco), 2.3 g/l KH<sub>2</sub>PO<sub>4</sub> (separate sterilization), 12.5 g/l K<sub>2</sub>HPO<sub>4</sub> (separate sterilization) and 50 µg/ml ampicillin and cultured at 30°C for 4 hours and then at 40°C for 3 hours, under conditions of 600 rpm and 2.5 L/min of aeration.

15 During the culturing, pH of the medium was maintained at 7.0 using 28% aqueous ammonia. In addition, glucose was added during the culturing when required. The culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

20 *Corynebacterium ammoniagenes* ATCC 21170 was inoculated into a 300 ml-baffled conical flask containing 20 ml of an aqueous medium of 50 g/l glucose, 10 g/l Polypeptone (manufactured by Nippon Seiyaku), 10 g/l Yeast Extract (manufactured by Oriental Yeast), 5 g/l urea, 5 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/l KH<sub>2</sub>PO<sub>4</sub>, 3 g/l K<sub>2</sub>HPO<sub>4</sub>, 1 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1 g/l CaCl<sub>2</sub> · 2H<sub>2</sub>O, 10 mg/l FeSO<sub>4</sub> · 7H<sub>2</sub>O, 10 mg/l ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 20 mg/l MnSO<sub>4</sub> · 4-6H<sub>2</sub>O, 20 mg/l L-cysteine, 10 mg/l calcium D-pantothenate, 5 mg/l vitamin B<sub>1</sub>, 5 mg/l nicotinic acid and 30 µg/l biotin (adjusted to pH 7.2 with 10 N NaOH) and cultured at 28°C for 24 hours under shaking at 220 rpm.

25 A 20 ml portion of the culture broth was inoculated into a 2 L baffled conical flask containing 250 ml of the same aqueous medium and cultured at 28°C for 24 hours under shaking at 220 rpm. The thus obtained culture broth was used as a seed culture broth.

30 A 250 ml portion of the seed culture broth was inoculated into a 5 L culture vessel containing 2.25 L of an aqueous medium of 150 g/l glucose, 5 g/l meat extract (manufactured by Kyokuto Pharmaceutical Industry), 10 g/l KH<sub>2</sub>PO<sub>4</sub>, 10 g/l K<sub>2</sub>HPO<sub>4</sub>, 10 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1 g/l CaCl<sub>2</sub> · 2H<sub>2</sub>O, 20 mg/l FeSO<sub>4</sub> · 7H<sub>2</sub>O, 10 mg/l ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 20 mg/l g/l MnSO<sub>4</sub> · 4-6H<sub>2</sub>O (separate sterilization), 15 mg/l β-alanine (separate sterilization), 20 mg/l L-cysteine, 100 µg/l biotin, 2 g/l urea and 5 mg/l vitamin B<sub>1</sub> (separate sterilization) (adjusted to pH 7.2 with 10 N NaOH) and cultured at 32°C for 24 hours under conditions of 600 rpm and 2.5 L/min of aeration. During the culturing, pH of the culture broth was maintained at 6.8 using 28% aqueous ammonia.

35 The culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

40 A 30 ml portion of a reaction solution having a composition of 40 g/l *Escherichia coli* KY8415/pNT12 wet cells, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 100 g/l glucose, 20 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 21.2 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 21 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

45 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and glucose and KH<sub>2</sub>PO<sub>4</sub> were added when required.

45 By the reaction, 43.9 g/l UDP-Glc (2Na salt) was formed in the reaction solution.

Example 3: Construction of recombinant plasmid capable of expressing galT and galK

Construction process of recombinant plasmid pNT25 capable of expressing galT and galK is described in the following (Fig. 3).

50 The sense strand DNA primer shown in SEQ ID NO:5 and the antisense strand DNA primer shown in SEQ ID NO:6 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template, under the same conditions as described in the foregoing.

55 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20 µl of TE. Using 5 µl portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Hinc*II, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 2.3 kb was recovered using Gene Clean II Kit. A 0.2 µg portion of pBluescript II SK+ DNA was cleaved with restriction enzymes *Hind*III and *Eco*RV, the resulting DNA fragments were separated by agarose gel electrophoresis

and then a fragment of 3.0 kb was recovered in the same manner.

Using a ligation kit, the fragments of 2.3 kb and 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, plasmid pNT19 containing galT and galK genes was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 3).

A 0.5 µg portion of the pNT19 DNA was cleaved with restriction enzymes *C*laI and *B*amHI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 2.3 kb was recovered in the same manner.

10 A 0.2 µg portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *C*laI and *B*amHI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered in the same manner.

Using a ligation kit, the fragments of 2.3 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

15 Using the ligation reaction solution, *Escherichia coli* NM52 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, plasmid pNT25 capable of expressing galT and galK simultaneously was obtained. Structure of the 20 plasmid was recognized by restriction enzyme cleavage (Fig. 3).

#### Example 4: Production of UDP-Gal

##### 1) Preparation of galT, galK, galU, ppa expression strain

25 Using the pNT32 DNA obtained in Example 1-3), *Escherichia coli* NM522/pNT25 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and 10 µg/ml chloramphenicol and then cultured overnight at 30°C. By selecting the thus grown transformants, *Escherichia coli* NM522/pNT25/pNT32 was obtained as the galT, galK, galU, ppa expression strain.

30 2) Production of UDP-Gal

Escherichia coli NM522/pNT25/pNT32 obtained in Example 4-1) was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. Also, *Corynbacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, the wet cells can be preserved at -20°C and utilized by thawing them prior to use.

35 A 2 L portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pNT25/pNT32 wet cells, 150 g/l *Corynbacterium ammoniagenes* ATCC 21170 wet cells, 80 g/l glucose, 20 g/l galactose, 15 g/l *KH*<sub>2</sub>*PO*<sub>4</sub>, 5 g/l *MgSO*<sub>4</sub> • 7*H*<sub>2</sub>O, 5 g/l phytic acid, 21.2 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into 5 L culture vessel, and 26 hours of the reaction was carried out at 32°C under stirring the reaction solution at 600 rpm with an aeration rate of 1 L/min.

40 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and glucose, galactose and *KH*<sub>2</sub>*PO*<sub>4</sub> were added when required.

By the reaction, 47.4 g/l UDP-Gal (2Na salt) was formed in the reaction solution.

45 Example 5: Construction of recombinant plasmid capable of expressing galT and galK in *Corynbacterium ammoniagenes*

Construction process of recombinant plasmid pTK7 capable of expressing *Escherichia coli*-derived galT and galK in *Corynbacterium ammoniagenes* is described in the following (Fig. 4).

##### 1) Construction of pCG116

50 Plasmid pCG116 capable of replicating in *Corynbacterium ammoniagenes* was constructed in the following manner.

55 A 0.5 µg portion of plasmid pCG11 (Japanese Published Examined Patent Application No. 91827/94) DNA was cleaved with restriction enzymes *Pst*I and *Stu*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 6.5 kb was recovered using Gene Clean II Kit.

On the other hand, a 1.0  $\mu$ g portion of plasmid pUC19 DNA was cleaved with a restriction enzyme *Eco*RI and then blunt-ended using DNA Blunting Kit (manufactured by Takara Shuzo Co., Ltd.). The DNA thus blunt-ended was cleaved with a restriction enzyme *Pst*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 43 bp was recovered using MERmaid Kit (manufactured by Bio101).

5 Using a ligation kit, the fragments of 6.5 kb and 43 bp were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Corynebacterium ammoniagenes* ATCC 21170 was transformed by the electroporation method (*FEMS Microbiol. Lett.*, 65, 299 (1989)), and the resulting transformant was spread on LB agar medium containing 100  $\mu$ g/ml spectinomycin and then cultured at 30°C for 2 days.

10 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the known process (*J. Bacteriol.*, 159, 306 (1984)), plasmid pCG116 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 4).

## 2) Construction of pTK7 capable of expressing galT and galK

15 A 1.0  $\mu$ g portion of the galT and galK expression plasmid pNT25 DNA obtained in Example 3 was cleaved with restriction enzymes *Xba*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.5 kb was recovered using Gene Clean II Kit.

On the other hand, a 0.5  $\mu$ g portion of the plasmid pCG116 DNA prepared in Example 5-1) was cleaved with a restriction enzymes *Sac*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 6.5 kb was recovered in the same manner.

20 Using a ligation kit, the fragments of 3.5 kb and 6.5 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Corynebacterium ammoniagenes* ATCC 21170 was transformed by the electroporation process, and the resulting transformant was spread on LB agar medium containing 100  $\mu$ g/ml spectinomycin and then cultured at 30°C for 2 days.

25 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the known process, plasmid pTK7 capable of expressing galT and galK simultaneously was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 4).

## Example 6: Production of UDP-Gal

30 *Corynebacterium ammoniagenes* ATCC 21170/pTK7 obtained in Example 5 was cultured by the same process as described in Example 2 at 32°C for 20 hours and then at 40°C for 4 hours, and the thus obtained culture broth was centrifuged to obtain wet cells. As occasion demands, the wet cells can be preserved at -20°C and utilized by thawing them prior to use.

35 A 30 ml portion of a reaction solution having a composition of 150 g/l *Corynebacterium ammoniagenes* ATCC 21170/pTK7 wet cells, 40 g/l fructose, 20 g/l galactose, 15 g/l  $KH_2PO_4$ , 5 g/l  $MgSO_4 \cdot 7H_2O$ , 5 g/l phytic acid, 10.6 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 22 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and fructose, galactose and

40  $KH_2PO_4$  were added when required.

By the reaction, 7.2 g/l UDP-Gal (2Na salt) was formed in the reaction solution.

## Example 7: Construction of glmU, ppa, pgm, glmM, glk and ptkB expression plasmid

### 1) Construction of glmU and ppa expression plasmid

45 The sense strand DNA primer shown in SEQ ID NO:7 and the antisense strand DNA primer shown in SEQ ID NO:8 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template, under the same conditions as described in the foregoing.

50 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The DNA precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.4 kb was recovered using Gene Clean II Kit. A 0.5  $\mu$ g portion of pPA31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.2 kb was recovered in the same manner.

55 Using a ligation kit, the fragments of 1.4 kb and 4.2 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* KY8415 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin

and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, *glmU* expression plasmid pNT10 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 5).

5 A 0.5 µg portion of the pNT12 DNA obtained in Example 1-3) was cleaved with restriction enzymes *Bam*HI and *Sa*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.0 kb was recovered in the same manner. A 0.2 µg portion of the just described pNT10 DNA was cleaved with restriction enzymes *Bam*HI and *Sa*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.3 kb was recovered in the same manner.

10 Using a ligation kit, the fragments of 1.0 kb and 5.3 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* KY8415 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

15 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, the *glmU, ppa* simultaneous expression plasmid pNT14 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 5).

## 2) Construction of *pgm* expression plasmid

20 The sense strand DNA primer shown in SEQ ID NO:9 and the antisense strand DNA primer shown in SEQ ID NO:10 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template, under the same conditions as described in the foregoing.

After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20 µl of TE. Using 5 µl portion of the solution, the DNA was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.8 kb was recovered using Gene Clean II Kit. A 0.2 µg portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered in the same manner.

25 Using a ligation kit, the fragments of 1.8 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

30 Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

35 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, *pgm* expression plasmid pNT24 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 6).

## 3) Construction of *glmM* expression plasmid

40 The sense strand DNA primer shown in SEQ ID NO:11 and the antisense strand DNA primer shown in SEQ ID NO:12 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Escherichia coli* W3110 as the template, under the same conditions as described in the foregoing.

45 After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20 µl of TE. Using 5 µl portion of the solution, the DNA was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.6 kb was recovered using Gene Clean II Kit. A 0.2 µg portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered in the same manner.

50 Using a ligation kit, the fragments of 1.6 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the above-mentioned known process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

55 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, *glmM* expression plasmid pNT44 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 7).

## 4) Construction of *glk* expression plasmid

The sense strand DNA primer shown in SEQ ID NO:13 and the antisense strand DNA primer shown in SEQ ID

NO:14 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Escherichia coli* W3110 as the template, under the same conditions as described in the foregoing.

After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 0.5 kb was recovered using Gene Clean II Kit.

5 A 0.2  $\mu$ g portion of the pPA31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.2 kb was recovered in the same manner.

10 Using a ligation kit, the fragments of 0.5 kb and 4.2 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

15 By extracting a plasmid from the thus grown colonies of the transformant in the usual way, plasmid pNT45 containing a part of *glk* was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 8).

The PCR was carried out under the same conditions described above, the DNA contained in 5  $\mu$ l portion of the thus obtained DNA solution was cleaved with a restriction enzyme *Hind*III, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 0.5 kb was recovered in the same manner. A 0.2  $\mu$ g portion of the pNT45 DNA obtained by the just described process was cleaved with the restriction enzyme *Hind*III and subjected to a 20 dephosphorylation treatment with alkaline phosphatase, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.7 kb was recovered in the same manner.

Using a ligation kit, the fragments of 0.5 kb and 4.7 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

25 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual way, *glk* expression plasmid pNT46 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 8).

### 30 5) Construction of *pfkB* expression plasmid

The sense strand DNA primer shown in SEQ ID NO:15 and the antisense strand DNA primer shown in SEQ ID NO:16 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of the strain W3110 as the template, under the same conditions as described in the foregoing.

35 After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Eco*RV, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.3 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pBluescript II SK+ DNA was cleaved with restriction enzymes *Hind*III and *Eco*RV, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

40 Using a ligation kit, the fragments of 1.3 kb and 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

45 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plasmid pNT43 containing the *pfkB* gene was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 9).

A 0.5  $\mu$ g portion of the pNT43 DNA was cleaved with restriction enzymes *Cla*I and *Sac*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.3 kb was recovered in the same manner.

50 A 0.2  $\mu$ g portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Sac*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.7 kb was recovered in the same manner.

Using a ligation kit, the fragments of 1.3 kb and 5.7 kb were subjected to ligation reaction for 16 hours at 16°C.

55 Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process,

pfkB expression plasmid pNT47 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 9).

Example 8: Production of UDP-GlcNAc

5 *Escherichia coli* KY8415/pNT14, NM522/pNT24, NM522/pNT44 and NM522/pNT47 obtained in Example 7 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths were centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

10 A 0.1 ml portion of a reaction solution having a composition of 6 g/l *Escherichia coli* NM522/pNT24 wet cells, 6 g/l NM522/pNT47 wet cells, 100 mM Tris-HCl buffer (pH 8.0), 6 mM MgCl<sub>2</sub> • 6H<sub>2</sub>O, 10 mM glucose-6-phosphate, 2.5 mM fructose-6-phosphate, 2.5 mM ATP and 4 g/l Nymeen S-215 was put into a 1.5 ml tube, and 1 hour of the reaction was carried out at 37°C. The reaction solution was treated at 65°C for 5 minutes and shortage in cells and substances was suspended until 0.3 g/l *Escherichia coli* KY8415/pNT14 wet cells, 6 g/l NM522/pNT44 wet cells, 5 mM glucosamine-6-phosphate, 5 mM acetyl-CoA and 5 mM UTP, and then 30 minutes of the reaction was carried out at 37°C to find that 15 2.5 mM (1.6 g/l) UDP-GlcNAc (2Na salt) was formed in the reaction solution. In this connection, when *Escherichia coli* 2.5 mM (1.6 g/l) UDP-GlcNAc (2Na salt) was not added, formed amounts of the UDP-GlcNAc were 0.08 NM522/pNT24 wet cells or NM522/pNT47 wet cells were not added, formed amounts of the UDP-GlcNAc were 0.08 mM and 0.16 mM, respectively.

These results indicate that Glc-1,6-P<sub>2</sub> necessary for the expression of glmM activity can be provided by the combination of a pgm expression strain with a pfkB expression strain.

20 Example 9: Production of UDP-GlcNAc

Escherichia coli KY8415/pNT14, NM522/pNT24, NM522/pNT44, NM522/pNT46 and NM522/pNT47 obtained in Example 7 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynebacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the thus obtained culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

A 30 ml portion of a reaction solution having a composition of 10 g/l wet cells of each of *Escherichia coli* KY8415/pNT14, NM522/pNT24, NM522/pNT44, NM522/pNT46, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 50 g/l fructose, 80 g/l glucosamine hydrochloride, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> • 7H<sub>2</sub>O, 5 g/l phytic acid, 10 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 10 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and fructose and KH<sub>2</sub>PO<sub>4</sub> were added when required.

By the reaction, 6.2 g/l UDP-GlcNAc (2Na salt) was formed in the reaction solution.

Example 10: Construction of galK expression plasmid

40 A 0.5 µg portion of the pNT25 DNA obtained in Example 3-1) was cleaved with restriction enzymes *Cla*I and *Eco*RV, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 6.7 kb was recovered using Gene Clean II kit. The thus recovered DNA was blunt-ended using DNA Blunting Kit and then subjected to ligation reaction for 16 hours at 16°C using a ligation kit.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, galK expression plasmid pNT54 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 10).

50 Example 11: Production of UDP-GlcNAc

Escherichia coli NM522/pNT54 obtained in Example 10 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. Also, *Corynebacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

A 30 ml portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pNT54 wet cells, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 40 g/l fructose, 67 g/l N-acetylglucosamine, 15 g/l KH<sub>2</sub>PO<sub>4</sub>.

5 g/l  $MgSO_4 \cdot 7H_2O$ , 5 g/l phytic acid, 10 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 27 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and fructose and  $KH_2PO_4$  were added when required.

By the reaction, 17.1 g/l UDP-GlcNAc (2Na salt) was formed in the reaction solution.

Example 12: Simultaneous production of UDP-GlcNAc and UDP-Gal

10 The strain NM522/pNT25 obtained in Example 3 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. Also, *Corynbacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

15 A 30 ml portion of a reaction solution having a composition of 25 g/l *Escherichia coli* NM522/pNT25 wet cells, 150 g/l *Corynbacterium ammoniagenes* ATCC 21170 wet cells, 60 g/l fructose, 50 g/l N-acetylglucosamine, 40 g/l galactose, 15 g/l  $KH_2PO_4$ , 5 g/l  $MgSO_4 \cdot 7H_2O$ , 5 g/l phytic acid, 10 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 24 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and  $KH_2PO_4$  was added when required.

20 By the reaction, 11.4 g/l UDP-GlcNAc (2Na salt) and 18 g/l UDP-Gal (2Na salt) were formed in the reaction solution.

Example 13: Construction of plasmid capable of expressing manB, manC, pgm and pfkB

25 1) Construction of manB, manC expression plasmid

The sense strand DNA primer shown in SEQ ID NO:17 and the antisense strand DNA primer shown in SEQ ID NO:18 were synthesized. The PCR, was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Escherichia coli* W3110 as the template, under the same conditions as described in the foregoing.

30 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pBluescript II SK+ DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

35 Using a ligation kit, both of the fragments of 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

40 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the above-mentioned known process, plasmid pNK6 containing manC and manB was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig.11).

45 A 0.5  $\mu$ g portion of the pNK6 DNA was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered. A 0.2  $\mu$ g portion of pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered.

50 Using a ligation kit, the fragments of 3.0 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

55 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, manC, manB expression plasmid pNK7 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 11).

2) Construction of pgm, pfkB simultaneous expression plasmid

A 0.5  $\mu$ g portion of the pNT24 DNA obtained in Example 7 was cleaved with restriction enzymes *Xba*I and *Bam*HI,

the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered using Gene Clean II Kit. On the other hand, a 0.2  $\mu$ g portion of pSTV28 DNA (manufactured by Takara Shuzo Co., Ltd.) was cleaved with restriction enzymes *Sall* and *BamHI*, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

5 Using a ligation kit, both of the fragments of 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 10  $\mu$ g/ml chloramphenicol and then cultured overnight at 30°C.

10 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plasmid pNT53 containing the *pgm* gene was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 12).

The sense strand DNA primer shown in SEQ ID NO:19 was synthesized, and the PCR was carried out using the sense strand DNA primer and the antisense strand DNA primer shown in SEQ ID NO:16, and the pNT47 DNA obtained in Example 7 as the template, under the same conditions as described in the foregoing.

15 After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *EcoRV* and *BglII*, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.3 kb was recovered in the same manner. A 0.2  $\mu$ g portion of pNT53 DNA was cleaved with restriction enzymes *SmaI* and *BamHI*, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 6.0 kb was recovered in the same manner.

20 Using a ligation kit, the fragments of 1.3 kb and 6.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 10  $\mu$ g/ml chloramphenicol and cultured overnight at 30°C.

25 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plasmid pNT55 capable of expressing *pgm* and *pfkB* was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 12).

#### Example 14: Production of GDP-Man

##### 30 1) Preparation of *manB*, *manC*, *pgm*, *pfkB* expression strain

Using the pNT55 DNA obtained in Example 13-2), *Escherichia coli* NM522/pNK7 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and 10  $\mu$ g/ml chloramphenicol and cultured overnight at 30°C. By selecting the thus grown transformants, *Escherichia coli* NM522/pNK7/pNT55 was obtained as a *manB*, *manC*, *pgm*, *pfkB* expression strain.

##### 2) Production of GDP-Man

40 *Escherichia coli* NM522/pNK7/pNT55 obtained in the above step 1) and *Escherichia coli* NM522/ pNT46 obtained in Example 7 were cultured separately in the same manner as in Example 2, and each of thus obtained the culture broths was centrifuged to obtain wet cells. Also, *Corynbacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

45 A 30 ml portion of a reaction solution having a composition of 25 g/l *Escherichia coli* NM522/pNK7/pNT55 wet cells, 25 g/l the NM522/pNT46 wet cells, 150 g/l *Corynbacterium ammoniagenes* ATCC 21170 wet cells, 60 g/l fructose, 50 g/l mannose, 15 g/l  $\text{KH}_2\text{PO}_4$ , 5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g/l phytic acid, 60 g/l GMP (2Na, 7 $\text{H}_2\text{O}$  salt), 4 g/l Mymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 24 hours of the reaction was carried out under stirring the reaction solution with a magnetic stirrer (900 rpm).

50 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and  $\text{KH}_2\text{PO}_4$  was added when required.

By the reaction, 14.6 g/l GDP-Man (2Na, 1 $\text{H}_2\text{O}$  salt) was formed in the reaction solution.

#### Example 15: Construction of *gmd*, *wcaG* expression plasmid

55 The sense strand DNA primer shown in SEQ ID NO:20 and the antisense strand DNA primer shown in SEQ ID NO:21 were synthesized. The PCR, was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Escherichia coli* W3110 as the template, under the same conditions as described in the foregoing.

After completion of the PCR, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Xba*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 2.3 kb was recovered using Gene Clean II Kit.

5 A 0.2  $\mu$ g portion of the pPA31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Hind*III and *Sal*I, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.9 kb was recovered in the same manner.

Using a ligation kit, the fragments of 2.3 kb and 3.9 kb were subjected to ligation reaction for 16 hours at 16°C.

10 Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

15 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plasmid pNK8 containing *god* and *wcaG* was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 13).

16 Example 16: Production of GDP-Fuc

Escherichia coli NM522/pNK7/pNT55 obtained in Example 14, NM522/pNK8 obtained in Example 15 and NM522/pNT46 obtained in Example 7 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynbacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

20 A 30 ml portion of a reaction solution having a composition of 25 g/l *Escherichia coli* NM522/pNK7/pNT55 wet cells, 25 g/l *Escherichia coli* NM522/pNK8 wet cells, 25 g/l *Escherichia coli* NM522/pNT46 wet cells, 150 g/l *Corynbacterium ammoniagenes* ATCC 21170 wet cells, 40 g/l fructose, 60 g/l mannose, 15 g/l  $KH_2PO_4$ , 5 g/l  $MgSO_4 \cdot 7H_2O$ , 5 g/l phytic acid, 60 g/l GMP (2Na/7H<sub>2</sub>O salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 24 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

25 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and  $KH_2PO_4$  was added when required.

30 By the reaction, 1.0 g/l GDP-Fuc (2.5Na, 1H<sub>2</sub>O salt) was formed in the reaction solution.

Example 17: Construction of *neuA* expression plasmid

35 Chromosomal DNA of *Escherichia coli* K235 (ATCC 13027) was prepared in the same manner as in Example 1. The sense strand DNA primer shown in SEQ ID NO:22 and the antisense strand DNA primer shown in SEQ ID NO:23 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Escherichia coli* K235 (ATCC 13027) as the template, under the same conditions as described in the foregoing.

40 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Eco*RI and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.3 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pBluescript II SK+ DNA was cleaved with restriction enzymes *Eco*RI and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

45 Using a ligation kit, the fragments of 1.3 kb and 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

50 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plasmid pTA12 containing the *neuA* gene was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 14).

55 A 0.5  $\mu$ g portion of the pTA12 DNA was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.3 kb was recovered in the same manner. A 0.2  $\mu$ g portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered in the same manner.

Using a ligation kit, the fragments of 1.3 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process.

and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, neuA expression plasmid pTA14 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 14).

5 Example 18: Production of CMP-NeuAc

Escherichia coli NM522/pTA14 obtained in Example 17, C600/pNAL1 (*Appl. Environ. Microbiol.*, **51**, 562 (1986)) and JF646/pMW5 (*J. Biol. Chem.*, **261**, 5568 (1986)) were separately cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynbacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

10 A 30 ml portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pTA14 wet cells, 15 g/l *Escherichia coli* C600/pNAL1 wet cells, 25 g/l *Escherichia coli* JF646/pMW5 wet cells, 150 g/l *Corynbacterium ammoniagenes* ATCC 21170 wet cells, 10 g/l orotic acid (potassium salt), 20 g/l pyruvic acid (Na salt), 40 g/l fructose, 10 g/l N-acetylmannosamine, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 24 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

15 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and KH<sub>2</sub>PO<sub>4</sub> was added when required.

20 By the reaction, 2.7 g/l CMP-NeuAc (Na salt) was formed in the reaction solution.

Example 19: Production of lacto-N-tetraose

25 i) Preparation of  $\beta$ 1,3-galactosyltransferase

Namalwa cell line KJM-1 transformed with plasmid pAMoERSAW1 (Japanese Published Unexamined Patent Application No. 181759/94) containing a gene encoding a fusion protein of the IgG binding region of protein A with  $\beta$ 1,3-galactosyltransferase was suspended in 30 ml of RPMI640 · ITPSGF medium containing 0.5 mg/ml G418 (manufactured by Gibco), to a density of  $5 \times 10^4$  cells/ml, and cultured at 37°C for 8 days in a CO<sub>2</sub> incubator.

30 Cells were removed from the culture broth by centrifugation, and the supernatant was recovered. As occasion demands, the supernatant can be stored at -80°C and utilized by thawing the supernatant prior to use.

35 To the culture supernatant in which the fusion protein of the IgG binding region of protein A with  $\beta$ 1,3-galactosyltransferase has been formed were added sodium azide to a final concentration of 0.1% and then 50 µl of IgG Sepharose (manufactured by Pharmacia) which has been pre-treated in accordance with the manufacturer's instructions. The mixture was stirred overnight gently at 4°C.

40 After the stirring, the  $\beta$ 1,3-galactosyltransferase-linked IgG Sepharose was recovered by centrifugation and washed three times with 1 ml of RPMI640 · ITPSGF medium, and then the IgG Sepharose was used as the enzyme source of  $\beta$ 1,3-galactosyltransferase.

2) Production of lacto-N-tetraose

45 Lacto-N-neotetraose (manufactured by Oxford Glycosystems) was fluorescence-labeled by 2-aminopyridine in accordance with a known process (*Agric. Biol. Chem.*, **54**, 2169 (1990)) and mixed with 0.1 U  $\beta$ -galactosidase (manufactured by Seikagaku Kogyo K.K.), and then the mixture was allowed to react for 16 hours at 37°C to remove the galactose at the non-reducing end.

The reaction solution was heated at 100°C for 5 minutes to inactivate  $\beta$ -galactosidase.

50 GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc obtained by the reaction was used as a complex carbohydrate precursor, 0.5 U the  $\beta$ 1,3-galactosyltransferase linked IgG Sepharose obtained in the above step 1, 6 µl of a reaction solution containing UDP-Gal (5 mM) obtained in Example 4, 100 mM Tris-HCl (pH 7.9), 10 mM MnCl<sub>2</sub> and 2 mM  $\beta$ -mercaptoethanol was allowed to stand for 65 hours at 32°C to effect the reaction.

55 After completion of the reaction, amount of the product accumulated in the reaction solution was measured by HPLC under the following conditions:

Column:

TSK gel ODS-80TM column (4.6 mm × 30 cm, manufactured by TOSOH CORPOATION)

Liquid phase:  
0.02 M ammonium acetate buffer (pH 4.0)

Temperature:  
50°C

5 Flow rate:  
1 ml/min

Detection:  
Fluorescence detector (excitation wavelength 320 nm, radiation wavelength 400 nm)

10 Identification of the product was carried out by comparing elution time of aminopyridine-labeled lacto-N-tetraose with that of the labeled product.

By the reaction, 0.17 mM (0.12 g/l) lacto-N-tetraose was formed.

Example 20 : Production of lacto-N-neotetraose

15 In the same manner as in Example 19, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc was prepared from lacto-N-neotetraose and used as a complex carbohydrate precursor.

A 36  $\mu$ l portion of a reaction solution containing 0.5 mM of the complex carbohydrate precursor, 0.5 U  $\beta$ 1,4-galactosyltransferase (manufactured by Sigma), 6  $\mu$ l of a reaction solution containing UDP-Gal (5 mM) obtained in Example 20, 4, 100 mM Tris-HCl buffer (pH 7.9), 10 mM MnCl<sub>2</sub> and 2 mM  $\beta$ -mercaptoethanol was allowed to stand for 65 hours at 32°C to effect the reaction.

After completion of the reaction, amount of the product accumulated in the reaction solution was measured by HPLC under the same conditions of Example 19-2). Identification of the product was carried out by comparing elution time of the aminopyridine-labeled lacto-N-neotetraose with that of the product.

25 By the reaction, 0.15 mM (0.11 g/l) lacto-N-neotetraose was formed.

Example 21: Production of lacto-N-fucopentaose III

30 IgG Sepharose-linked  $\alpha$ 1,3-fucosyltransferase was prepared from namalwa cell line KJM-1 transformed with plasmid pAMoA-FT6 (*J. Biol. Chem.*, 269, 14730 (1994)) containing a gene encoding a fusion protein of the IgG binding region of protein A with  $\alpha$ 1,3-fucosyltransferase, in the same manner as in Example 19-1), and used as the enzyme source of  $\alpha$ 1,3-fucosyltransferase.

35 A 50  $\mu$ l portion of a reaction solution containing 0.25 mM lacto-N-neotetraose (manufactured by Oxford Glycosystems), 1.0 U of the IgG Sepharose-linked  $\alpha$ 1,3-fucosyltransferase, 6  $\mu$ l of a reaction solution containing GDP-Fuc (0.25 mM) obtained in Example 16, 100 mM Tris-HCl buffer (pH 7.9) and 10 mM MnCl<sub>2</sub> was allowed to stand for 24 hours at 37°C to effect the reaction.

After completion of the reaction, amount of the product accumulated in the reaction solution was measured using a sugar analyzer (DX-500) manufactured by Dionex. Identification of the product was carried out by comparing elution time of lacto-N-fucopentaose III (manufactured by Oxford Glycosystems) with that of the product.

40 By the reaction, 0.21 mM (0.18 g/l) lacto-N-fucopentaose III was formed.

Example 22: Construction of  $\alpha$ 1,4-galactosyltransferase (IgtC) expression plasmid

45 Chromosomal DNA of *Neisseria gonorrhoeae* (ATCC 33084) was prepared in the same manner as in Example 1. The sense strand DNA primer shown in SEQ ID NO:24 and the antisense strand DNA primer shown in SEQ ID NO:25 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *Neisseria gonorrhoeae* (ATCC 33084) as the template, under the same conditions as described in the foregoing.

50 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 1.0 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of the pPA31 DNA obtained in Example 1-1 was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 4.2 kb was recovered in the same manner.

55 Using a ligation kit, the fragments of 1.0 kb and 4.2 kb were subjected to ligation reaction for 16 hours at 16°C. Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual procedure, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, a IgIC expression plasmid pGT3 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 15).

5 Example 23 : Production of globotriose

Escherichia coli NM522/pNT25/pNT32 obtained in Example 4 and Escherichia coli NM522/pGT3 obtained in Example 22 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, Corynebacterium ammoniagenes ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

10 A 30 ml portion of a reaction solution having a composition of 50 g/l Escherichia coli NM522/pNT25/pNT32 wet cells, 50 g/l Escherichia coli NM522/pGT3 wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 100 g/l fructose, 100 g/l galactose, 100 g/l lactose, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 10 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 36 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

15 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and galactose, lactose, fructose and KH<sub>2</sub>PO<sub>4</sub> were added when required.

20 By the reaction, 188 g/l globotriose was formed in the reaction solution.

Cells were removed from the reaction solution by centrifugation, and a 10 ml portion of the thus obtained supernatant was purified by employing a process in which activated carbon was used, thereby obtaining 1.5 g of globotriose as white powder.

Example 24: Production of Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc

25 Escherichia coli NM522/pNT25/pNT32 obtained in Example 4 and Escherichia coli NM522/pGT3 obtained in Example 22 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, Corynebacterium ammoniagenes ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

30 A 30 ml portion of a reaction solution having a composition of 50 g/l Escherichia coli NM522/pNT25/pNT32 wet cells, 50 g/l Escherichia coli NM522/pGT3 wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 50 g/l fructose, 50 g/l galactose, 96 g/l N-acetyllactosamine, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 10 g/l orotic acid (potassium salt), 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 23 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

35 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and galactose, fructose and KH<sub>2</sub>PO<sub>4</sub> were added when required.

By the reaction, 10 g/l Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc was formed in the reaction solution.

Cells were removed from the reaction solution by centrifugation, and the formed product was purified from a 30 ml portion of the thus obtained supernatant by employing a process in which activated carbon was used, thereby obtaining 0.2 g of Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc as white powder.

Example 25: Construction of  $\beta$ 1,4-galactosyltransferase (IgtB) expression plasmid

40 The sense strand DNA primer shown in SEQ ID NO:26 and the antisense strand DNA primer shown in SEQ ID NO:27 were synthesized. The PCR was carried out using the synthesized DNA strands as primers, and the chromosomal DNA of *N. gonorrhoeae* (ATCC 33084) as the template, under the same conditions as described in the foregoing.

45 After completion of the PCR treatment, a precipitate of DNA was obtained by the ethanol precipitation process. The precipitate was dissolved in 20  $\mu$ l of TE. Using 5  $\mu$ l portion of the solution, the DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 0.8 kb was recovered using Gene Clean II Kit. A 0.2  $\mu$ g portion of pBluescript II SK+ DNA was cleaved with restriction enzymes *Hind*III and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 3.0 kb was recovered in the same manner.

50 Using a ligation kit, the fragments of 0.8 kb and 3.0 kb were subjected to ligation reaction for 16 hours at 16°C.

55 Using the ligation reaction solution, Escherichia coli NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml ampicillin and then cultured overnight at 30°C.

By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, plas-

mid pNT60P containing the *IgtB* gene was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 16).

5 A 0.5 µg portion of the pNT60P DNA was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 0.8 kb was recovered. A 0.2 µg portion of the pPAC31 DNA obtained in Example 1-1) was cleaved with restriction enzymes *Cla*I and *Bam*HI, the resulting DNA fragments were separated by agarose gel electrophoresis and then a fragment of 5.5 kb was recovered in the same manner.

10 Using a ligation kit, the fragments of 0.8 kb and 5.5 kb were subjected to ligation reaction for 16 hours at 16°C.

15 Using the ligation reaction solution, *Escherichia coli* NM522 was transformed in accordance with the usual process, and the resulting transformant was spread on LB agar medium containing 50 µg/ml ampicillin and then cultured overnight at 30°C.

20 By extracting a plasmid from the thus grown colonies of the transformant in accordance with the usual process, a *IgtB* expression plasmid pNT60 was obtained. Structure of the plasmid was recognized by restriction enzyme cleavage (Fig. 16).

25 Example 26: Production of N-acetyllactosamine

30 *Escherichia coli* NM522/pNT60 obtained in Example 25 and *Escherichia coli* NM522/pNT25 obtained in Example 3 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynebacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

25 A 30 ml portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pNT25 wet cells, 50 g/l *Escherichia coli* NM522/pNT60 wet cells, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 10 g/l orotic acid (potassium salt), 100 g/l fructose, 100 g/l N-acetylglucosamine, 100 g/l galactose, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 34 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

20 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and galactose, fructose and KH<sub>2</sub>PO<sub>4</sub> were added when required.

30 By the reaction, 114 g/l N-acetyllactosamine was formed in the reaction solution.

Example 27: Production of lactose

35 *Escherichia coli* NM522/pNT60 obtained in Example 25 and *Escherichia coli* NM522/pNT25 obtained in Example 3 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynebacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

40 A 30 ml portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pNT25 wet cells, 50 g/l *Escherichia coli* NM522/pNT60 wet cells, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 10 g/l orotic acid (potassium salt), 115 g/l glucose, 115 g/l galactose, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker, and 15 hours of the reaction was carried out at 32°C by stirring the reaction solution on a magnetic stirrer (900 rpm).

45 During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and KH<sub>2</sub>PO<sub>4</sub> was added when required.

50 By the reaction, 49 g/l lactose was formed in the reaction solution.

Example 28: Production of globotriose

55 *Escherichia coli* NM522/pNT60 obtained in Example 25, *Escherichia coli* NM522/pNT25 obtained in Example 3 and *Escherichia coli* NM522/pGT3 obtained in Example 22 were cultured in the same manner as in Example 2, and each of the thus obtained culture broths was centrifuged to obtain wet cells. Also, *Corynebacterium ammoniagenes* ATCC 21170 was cultured in the same manner as in Example 2, and the culture broth was centrifuged to obtain wet cells. As occasion demands, these wet cells can be preserved at -20°C and utilized by thawing the cells prior to use.

55 A 30 ml portion of a reaction solution having a composition of 50 g/l *Escherichia coli* NM522/pNT25 wet cells, 50 g/l *Escherichia coli* NM522/pNT60 wet cells, 50 g/l *Escherichia coli* NM522/pGT3 wet cells, 150 g/l *Corynebacterium ammoniagenes* ATCC 21170 wet cells, 10 g/l orotic acid (potassium salt), 115 g/l glucose, 115 g/l galactose, 15 g/l KH<sub>2</sub>PO<sub>4</sub>, 5 g/l MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200 ml beaker,

and 13 hours of the reaction was carried out at 32°C under stirring the reaction solution with a magnetic stirrer (900 rpm).

During the reaction, pH of the reaction solution was maintained at 7.2 using 4 N NaOH, and  $\text{KH}_2\text{PO}_4$  was added when required.

5 By the reaction, 5 g/l globotriose was formed in the reaction solution.

INDUSTRIAL APPLICABILITY

10 The present invention renders possible efficient industrial production of a sugar nucleotide from a nucleotide precursor and a sugar as the sole starting materials and of a complex carbohydrate from the sugar nucleotide and a complex carbohydrate precursor.

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## SEQUENCE LISTING

5

SEQ ID NO:1:

SEQUENCE LENGTH: 31

10

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

15

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

GGAGAAAGCT TATGGCTGCC ATTAATACGA A

31

20

SEQ ID NO:2:

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

30

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

AACACGGATC CGGATGTTAC TTCTTAATGC

30

SEQ ID NO:3:

40

SEQUENCE LENGTH: 28

SEQUENCE TYPE: nucleic acid

45

TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

50

SEQUENCE DESCRIPTION:

ATGGAGGATC CTGCTCTGTA TACCGTCT

28

55

SEQ ID NO:4:

5 SEQUENCE LENGTH: 20

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

10 MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

15 TGCTGGTCGA CCTGCGCTTG

20

SEQ ID NO:5:

20 SEQUENCE LENGTH: 31

SEQUENCE TYPE: nucleic acid

25 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

30 AAGGAAAGCT TATGACGCAA TTTAATCCCG T

31

35 SEQ ID NO:6:

SEQUENCE LENGTH: 20

SEQUENCE TYPE: nucleic acid

40 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

45 SEQUENCE DESCRIPTION:

50 GCAAAGTTAA CAGTCGGTAC

20

55

SEQ ID NO:7:

5 SEQUENCE LENGTH: 31

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

10 MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

15 TCAGGAAGCT TATGTTGAAT AATGCTATGA G

31

SEQ ID NO:8:

20 SEQUENCE LENGTH: 27

SEQUENCE TYPE: nucleic acid

25 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

30 TCTCCGGATC CCATGTGACC GGGTTAG

27

35 SEQ ID NO:9:

SEQUENCE LENGTH: 28

SEQUENCE TYPE: nucleic acid

40 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

45 SEQUENCE DESCRIPTION:

TCTAAATCGA TGCAGACAAA GGACAAAG

28

50 SEQ ID NO:10:

55

SEQUENCE LENGTH: 27

5 SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

10 SEQUENCE DESCRIPTION:

TTGCAGGATC CTCGTAGGCC TGATAAG

27

15

SEQ ID NO:11:

SEQUENCE LENGTH: 20

20 SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

25

SEQUENCE DESCRIPTION:

TGATATCCGC TCCCTTTCCG

20

30

SEQ ID NO:12:

SEQUENCE LENGTH: 26

35 SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

40 MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

45 ACAGCGGATC CGATGTGTTG GCTGAG

26

55 SEQ ID NO:13:

50 SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid  
5 TOPOLOGY: single-stranded  
MOLECULAR TYPE: other nucleic acid, synthetic DNA  
SEQUENCE DESCRIPTION:  
10 ACAGCAAGCT TTTGACTTTA GCGGAGCAG

29

15 SEQ ID NO:14:  
SEQUENCE LENGTH: 29  
SEQUENCE TYPE: nucleic acid  
20 TOPOLOGY: single-stranded  
MOLECULAR TYPE: other nucleic acid, synthetic DNA  
SEQUENCE DESCRIPTION:  
25 GAGTTGGATC CCGATATAAA AGGAAGGAT

29

30 SEQ ID NO:15:  
SEQUENCE LENGTH: 25.  
35 SEQUENCE TYPE: nucleic acid  
TOPOLOGY: single-stranded  
MOLECULAR TYPE: other nucleic acid, synthetic DNA  
40 SEQUENCE DESCRIPTION:  
45 TTTTAAGCT TCATTTATCA AGACT

25

45 SEQ ID NO:16:  
SEQUENCE LENGTH: 31  
50 SEQUENCE TYPE: nucleic acid

55

5 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

10 TTTTGATAT CCCCAATGCT GGGGGTTTT G

31

15 SEQ ID NO:17:

SEQUENCE LENGTH: 31

20 SEQUENCE TYPE: nucleic acid

25 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

30 CGTCAAAGCT TAAATGATAT TCGGGGATAA T

31

35 SEQ ID NO:18:

40 SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

45 TOPOLOGY: single-stranded

50 MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

45 AGGGAGGATC CGACATTACT CGTTC

25

55 SEQ ID NO:19:

SEQUENCE LENGTH: 33

SEQUENCE TYPE: nucleic acid

55 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

5 CCGCAAGATC TCGTAAAAAG GGTATCGATA AGC

33

10 SEQ ID NO:20:

SEQUENCE LENGTH: 27

15 SEQUENCE TYPE: nucleic acid

15 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

20 SEQUENCE DESCRIPTION:

TTGGGAAGCT TCCGGCAAAT GTGGTTT

27

25

SEQ ID NO:21:

SEQUENCE LENGTH: 25

30 SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

35 MOLECULAR TYPE: other nucleic acid, synthetic DNA

35 SEQUENCE DESCRIPTION:

40 ATAAACTCGA GAGAGACAAG CGGAG

25

40

SEQ ID NO:22:

45 SEQUENCE LENGTH: 27

SEQUENCE TYPE: nucleic acid

50 TOPOLOGY: single-stranded

50 MOLECULAR TYPE: other nucleic acid, synthetic DNA

55

## SEQUENCE DESCRIPTION:

5 TATTATCGAT GAATTAATAA TTTCATAG

27

SEQ ID NO:23:

10 SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

15 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

20 CTCTGGATCC AGTTACGTAT AATAT

25

25 SEQ ID NO:24:

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

30 TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

35 SEQUENCE DESCRIPTION:

30 CGGCAAGCTT ATTGTGCCTT TCCAATAAAA

40 SEQ ID NO:25:

SEQUENCE LENGTH: 28

45 SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

MOLECULAR TYPE: other nucleic acid, synthetic DNA

50 SEQUENCE DESCRIPTION:

55

ACTTGGATCC CCGTCAATAA ATCTTGCG

28

5

SEQ ID NO:26:

SEQUENCE LENGTH: 30

10

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

15

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

GGTAAAGCTT ATGCAAAACC AC GTTATCAG

30

20

SEQ ID NO:27:

SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid

TOPOLOGY: single-stranded

30

MOLECULAR TYPE: other nucleic acid, synthetic DNA

SEQUENCE DESCRIPTION:

35

AAACGGATCC TTATTGGAAA GGCACAATA

29

40

**Claims**

1. A process for producing a sugar nucleotide, which comprises:

45 selecting, as enzyme sources, a) a culture broth of a microorganism capable of producing nucleotide-5'-triphosphate (referred to as "NTP" hereinafter) from a nucleotide precursor, or a treated product of the culture broth, and b) a culture broth of a microorganism capable of producing a sugar nucleotide from a sugar and NTP, or a treated product of the culture broth; allowing the enzyme sources, the nucleotide precursor and the sugar to be present in an aqueous medium to form and accumulate the sugar nucleotide in the aqueous medium; and recovering the sugar nucleotide from the aqueous medium.

2. A process for producing a complex carbohydrate, which comprises:

55 selecting, as enzyme sources, a) a culture broth of a microorganism capable of producing nucleotide-5'-triphosphate (referred to as "NTP" hereinafter) from a nucleotide precursor, or a treated product of the culture broth, b) a culture broth of a microorganism capable of producing a sugar nucleotide from a sugar and NTP, or a treated product of the culture broth, and c) a culture broth of a microorganism, an animal cell or an insect cell

capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor, or a treated product of the culture broth; allowing the enzyme sources, the nucleotide precursor, the sugar and the complex carbohydrate precursor to be present in an aqueous medium to form and accumulate the complex carbohydrate in the aqueous medium; and recovering the complex carbohydrate from the aqueous medium.

3Gal, Gal $\alpha$ 1-4Gal, Gal $\alpha$ 1-3GalNAc, Gal $\alpha$ 1-4GalNAc, GlcNAc $\beta$ 1-3Gal, GlcNAc $\beta$ 1-4Gal, GlcNAc $\beta$ 1-6Gal, GlcNAc $\beta$ 1-3Glc, GlcNAc $\beta$ 1-4Glc, GlcNAc $\beta$ 1-3GlcNAc, GlcNAc $\beta$ 1-4GlcNAc, GlcNAc $\beta$ 1-6GalNAc, GlcNAc $\beta$ 1-2Man, GlcNAc $\beta$ 1-4Man, GlcNAc $\beta$ 1-6Man, GalNAc $\beta$ 1-3Gal, GalNAc $\beta$ 1-4Gal, GalNAc $\beta$ 1-4GlcNAc, GalNAc $\alpha$ 1-3GalNAc, Man $\beta$ 1-4GlcNAc, Man $\alpha$ 1-6Man, Man $\alpha$ 1-3Man, Man $\alpha$ 1-2Man, GlcUA $\beta$ 1-4GlcN, GlcUA $\beta$ 1-3Gal, GlcUA $\beta$ 1-3GlcNAc, GlcUA $\beta$ 1-3GalNAc, NeuAc $\alpha$ 2-3Gal, NeuAc $\alpha$ 2-6Gal, NeuAc $\alpha$ 2-3GlcNAc, NeuAc $\alpha$ 2-6GlcNAc, NeuAc $\alpha$ 2-3GalNAc, NeuAc $\alpha$ 2-6GalNAc, NeuAc $\alpha$ 2-8NeuAc, Fuc $\alpha$ 1-3Glc, Fuc $\alpha$ 1-4Glc, Fuc $\alpha$ 1-3GlcNAc, Fuc $\alpha$ 1-4GlcNAc, Fuc $\alpha$ 1-2Gal and Fuc $\alpha$ 1-6GlcNAc, or a complex carbohydrate which contains the complex carbohydrate.

5 11. The process according to claim 9 or 10, wherein the number of sugars contained in the complex carbohydrate is 10 or below.

10 12. The process according to claim 9 or 10, wherein the number of sugars contained in the complex carbohydrate is 6 or below.

15 13. The process according to claim 2 or 3, wherein the complex carbohydrate precursor is a complex carbohydrate precursor selected from monosaccharides, oligosaccharides, proteins, peptides, lipids, glycoproteins, glycolipids, glycopeptides and steroid compounds.

20 14. The process according to claim 13, wherein the complex carbohydrate precursor is a complex carbohydrate precursor selected from glucose, galactose, mannose, sialic acid, N-acetylglucosamine, N-acetylgalactosamine, lactose, N-acetyllactosamine, lacto-N-biose, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc, GlcNAc $\beta$ 1-4Gal $\beta$ 1-4Glc, globotriose, Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc, 2'-fucosyllactose, 3'-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-N-acetyllactosamine, 6'-sialyl-N-acetyllactosamine, sialylacto-N-biose, Lewis X, Lewis a, lacto-N-tetraose, lacto-N-neotetraose, 25 amine, 6'-sialyl-N-acetyllactosamine, sialylacto-N-biose, Lewis X, Lewis a, lacto-N-fucopentaose I, lacto-N-fucolactodifucotetraose, 3'-sialyl-3-fucosyllactose, sialyl-Lewis X, sialyl-Lewis a, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, LS-tetrasaccharide a, LS-tetrasaccharide b, LS-tetrapentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, LS-tetrasaccharide b, LS-tetrasaccharide c, (2,3)sialyllacto-N-neotetraose, and derivatives thereof, and ceramide and derivatives thereof, or a complex carbohydrate precursor containing the complex carbohydrate precursor.

30 15. The process according to claim 1 or 2, wherein the microorganism capable of producing NTP from a nucleotide precursor is a microorganism selected from microorganisms belonging to the genus *Corynebacterium*.

16. The process according to claim 15, wherein the microorganism belonging to the genus *Corynebacterium* belongs to *Corynebacterium ammoniagenes*.

35 17. The process according to claim 1 or 2, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP comprises at least one kind of microorganism.

18. The process according to claim 17, wherein at least one microorganism is selected from microorganisms belonging to the genus *Escherichia* and the genus *Corynebacterium*.

40 19. The process according to claim 18, wherein the microorganism belonging to the genus *Escherichia* is *Escherichia coli*.

20. The process according to claim 18, wherein the microorganism belonging to the genus *Corynebacterium* is *Corynebacterium ammoniagenes*.

45 21. The process according to claim 1 or 2, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from glucokinase (referred to as "glk" hereinafter), phosphoglcomutase (referred to as "pgm" hereinafter), glucose-1-phosphate uridylyltransferase (referred to as "galU" hereinafter) and pyrophosphatase (referred to as "ppa" hereinafter).

50 22. The process according to claim 21, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a glk-encoding gene, a pgm-encoding gene, a galU-encoding gene and a ppa-encoding gene.

55 23. The process according to claim 22, wherein the glk-encoding gene, the pgm-encoding gene, the galU-encoding gene and the ppa-encoding gene are genes derived from *Escherichia coli*.

24. The process according to claim 21, wherein the sugar nucleotide is uridine diphosphoglucose.

25. The process according to claim 21, wherein the microorganism is a microorganism having strong uridine diphosphoglucose dehydrogenase activity, and the sugar nucleotide is uridine diphosphoglucuronic acid.

5 26. The process according to claim 1 or 2, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong galactokinase (referred to as "galK" hereinafter) activity.

10 27. The process according to claim 26, wherein N-acetylglucosamine-1-phosphate is provided by the microorganism of claim 26 having strong galK activity using N-acetylglucosamine as a substrate.

28. The process according to claim 26, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains a galK-encoding gene.

15 29. The process according to claim 28, wherein the galK-encoding gene is a gene derived from *Escherichia coli*.

30. The process according to claim 26, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong galactose-1-phosphate uridylyltransferase (referred to as "galT" hereinafter) activity.

20 31. The process according to claim 30, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains a galT-encoding gene.

32. The process according to claim 31, wherein the galT-encoding gene is a gene derived from *Escherichia coli*.

25 33. The process according to claim 30, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from glucokinase (referred to as "glk" hereinafter), phosphoglucomutase (referred to as "pgm" hereinafter), glucose-1-phosphate uridylyltransferase (referred to as "galU" hereinafter) and pyrophosphatase (referred to as "ppa" hereinafter).

30 34. The process according to claim 33, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a glk-encoding gene, a pgm-encoding gene, a galU-encoding gene and a ppa-encoding gene.

35 35. The process according to claim 34, wherein the glk-encoding gene, the pgm-encoding gene, the galU-encoding gene and the ppa-encoding gene are genes derived from *Escherichia coli*.

36. The process according to claim 30 or 33, wherein the sugar nucleotide is uridine diphosphogalactose.

40 37. The process according to claim 26, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong N-acetylglucosamine-1-phosphate uridylyltransferase (referred to as "glmU" hereinafter) activity.

45 38. The process according to claim 37, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains a glmU-encoding gene.

39. The process according to claim 38, wherein the glmU-encoding gene is a gene derived from *Escherichia coli*.

50 40. The process according to claim 1 or 2, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong phosphoglucomutase (referred to as "pgm" hereinafter) and phosphofructokinase (referred to as "pfkB" hereinafter) activities.

55 41. The process according to claim 40, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a pgm-encoding gene and a pfkB-encoding gene.

42. The process according to claim 41, wherein the pgm-encoding gene and the pfkB-encoding gene are genes derived from *Escherichia coli*.

43. The process according to claim 40, wherein glucose-1,6-diphosphate is provided by the microorganism of claim 40 having strong pgm and pfkB activities using glucose-6-phosphate and fructose-6-phosphate as a substrate.
44. The process according to claim 43, wherein phosphoglucosamine mutase or phosphomannomutase activity is increased by glucose-1,6-diphosphate provided by the process of claim 43.
45. The process according to claim 40, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from glucosamine-1-phosphate acetyltransferase, N-acetylglucosamine-1-phosphate uridyltransferase (referred to as "glmU" hereinafter), pyrophosphatase (referred to as "ppa" hereinafter), phosphoglucosamine mutase (referred to as "glmM" hereinafter) and glucokinase (referred to as "glk" hereinafter).
46. The process according to claim 45, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a glmU-encoding gene, a ppa-encoding gene, a glmM-encoding gene and a glk-encoding gene.
47. The process according to claim 46, wherein the glmU-encoding gene, the ppa-encoding gene, the glmM-encoding gene and the glk-encoding gene are genes derived from *Escherichia coli*.
48. The process according to any one of claims 26, 37, 40 and 45, wherein the sugar nucleotide is uridine diphospho-N-acetylglucosamine.
49. The process according to any one of claims 26, 37, 40 and 45, wherein the microorganism is a microorganism having strong UDP-GlcNAc 4-epimerase activity and the sugar nucleotide is uridine diphospho-N-acetylgalactosamine.
50. The process according to claim 40, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from phosphomannomutase (referred to as "manB" hereinafter), mannose-1-phosphate guanylyltransferase (referred to as "manC" hereinafter) and glucokinase (referred to as "glk" hereinafter).
51. The process according to claim 50, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a manB-encoding gene, a manC-encoding gene and a glk-encoding gene.
52. The process according to claim 51, wherein the manB-encoding gene, the manC-encoding gene and the glk-encoding gene are genes derived from *Escherichia coli*.
53. The process according to claim 40 or 50, wherein the sugar nucleotide is guanosine diphosphomannose.
54. The process according to claim 40, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from phosphomannomutase (referred to as "manB" hereinafter), mannose-1-phosphate guanylyltransferase (referred to as "manC" hereinafter), glucokinase (referred to as "glk" hereinafter), GDP-4,6-mannose dehydratase (referred to as "gmd" hereinafter) and GDP-4-keto-6-deoxymannose epimerase/reductase (referred to as "wcaG" hereinafter).
55. The process according to claim 54, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a manB-encoding gene, a manC-encoding gene, a glk-encoding gene, a gmd-encoding gene and a wcaG-encoding gene.
56. The process according to claim 55, wherein the manB-encoding gene, the manC-encoding gene, the glk-encoding gene, the gmd-encoding gene and the wcaG-encoding gene are genes derived from *Escherichia coli*.
57. The process according to claim 40 or 54, wherein the sugar nucleotide is guanosine diphosphofucose.
58. The process according to claim 1 or 2, wherein the microorganism capable of producing a sugar nucleotide from a sugar and NTP is a microorganism having strong activity of at least one enzyme selected from GlcNAc 2-epimerase

rase, CMP-NeuAc synthetase (referred to as "neuA" hereinafter), NeuAc aldolase (referred to as "nanA" hereinafter), NeuAc synthetase (referred to as "neuB" hereinafter) and CTP synthetase (referred to as "pyrG" hereinafter).

59. The process according to claim 58, wherein the microorganism comprises at least one microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains at least one gene selected from a GlcNAc 2-epimerase-encoding gene, a neuA-encoding gene, a nanA-encoding gene, a neuB-encoding gene and a pyrG-encoding gene.

60. The process according to claim 59, wherein the neuA-encoding gene, the nanA-encoding gene, the neuB-encoding gene and the pyrG-encoding gene are genes derived from *Escherichia coli*.

61. The process according to claim 58, wherein the sugar nucleotide is cytidine monophospho-N-acetylneuraminic acid.

62. The process according to claim 2 or 3, wherein the microorganism capable of producing a complex carbohydrate from a sugar nucleotide and a complex carbohydrate precursor belongs to *Escherichia coli*, *Saccharomyces cerevisiae* or *Corynebacterium ammoniagenes*.

63. The process according to claim 62, wherein the microorganism is a microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains a gene encoding a transferase selected from glucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyltransferase, sialyltransferase and fucosyltransferase.

64. The process according to claim 63, wherein the gene encoding a transferase selected from glucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyltransferase, sialyltransferase and fucosyltransferase is derived from a microorganism.

65. The process according to claim 2 or 3, wherein the animal cell is COS-7 cell or namalwa KJM-1 cell, and the insect cell is Sf9 cell.

66. The process according to claim 2 or 3, wherein the animal cell or insect cell is an animal cell or insect cell having a recombinant DNA comprising a vector and a DNA fragment which contains a gene encoding a transferase selected from glucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyltransferase, sialyltransferase and fucosyltransferase.

67. The process according to claim 66, wherein the gene encoding a transferase selected from glucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, glucuronosyltransferase, mannosyltransferase, sialyltransferase and fucosyltransferase is derived from an animal cell.

68. A process for producing N-acetylglucosamine 1-phosphate, which comprises:

selecting, as an enzyme source, a culture broth of the microorganism of claim 26 having strong galK activity, or a treated product of the culture broth;

allowing the enzyme source and N-acetylglucosamine to be present in an aqueous medium to form and accumulate N-acetylglucosamine-1-phosphate in the aqueous medium, and

recovering the N-acetylglucosamine-1-phosphate from the aqueous medium.

69. The process according to claim 68, wherein the microorganism is a microorganism having a recombinant DNA comprising a vector and a DNA fragment which contains a galK-encoding gene.

70. The process according to claim 69, wherein the galK-encoding gene is a gene encoding galactokinase derived from *Escherichia coli*.

71. The process according to claim 68, wherein the treated product of the culture broth is a concentrated product of the culture broth, a dried product of the culture broth, a culture supernatant obtained by centrifuging the culture broth, a concentrated product of the culture supernatant, an enzyme preparation obtained from the culture supernatant, cells obtained by centrifuging the culture broth, a dried product of the cells, a freeze-dried product of the cells, a surfactant-treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product

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of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells, an immobilized product of the cells, or an enzyme preparation obtained by extraction from the cells.

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FIG.1

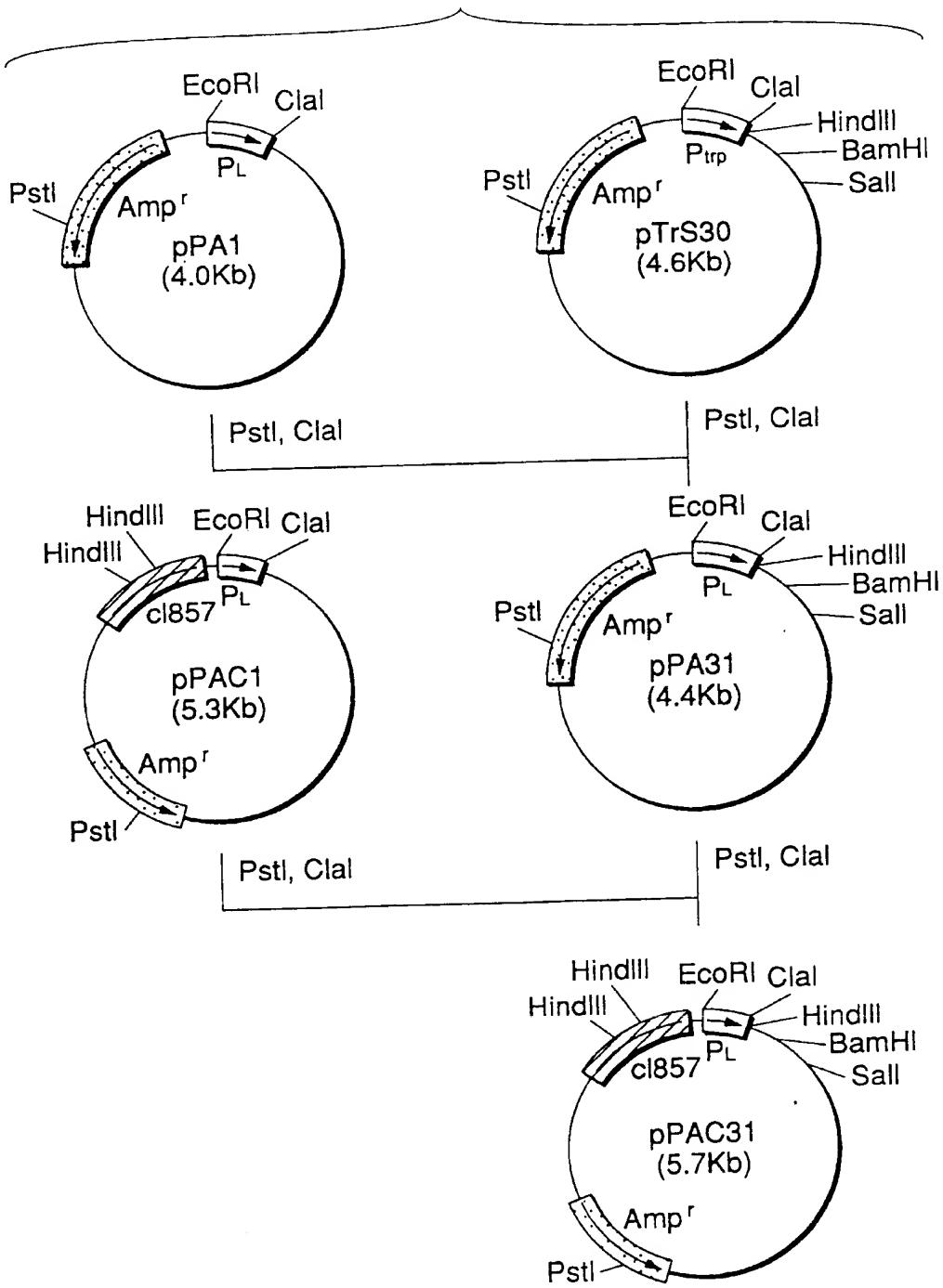


FIG.2

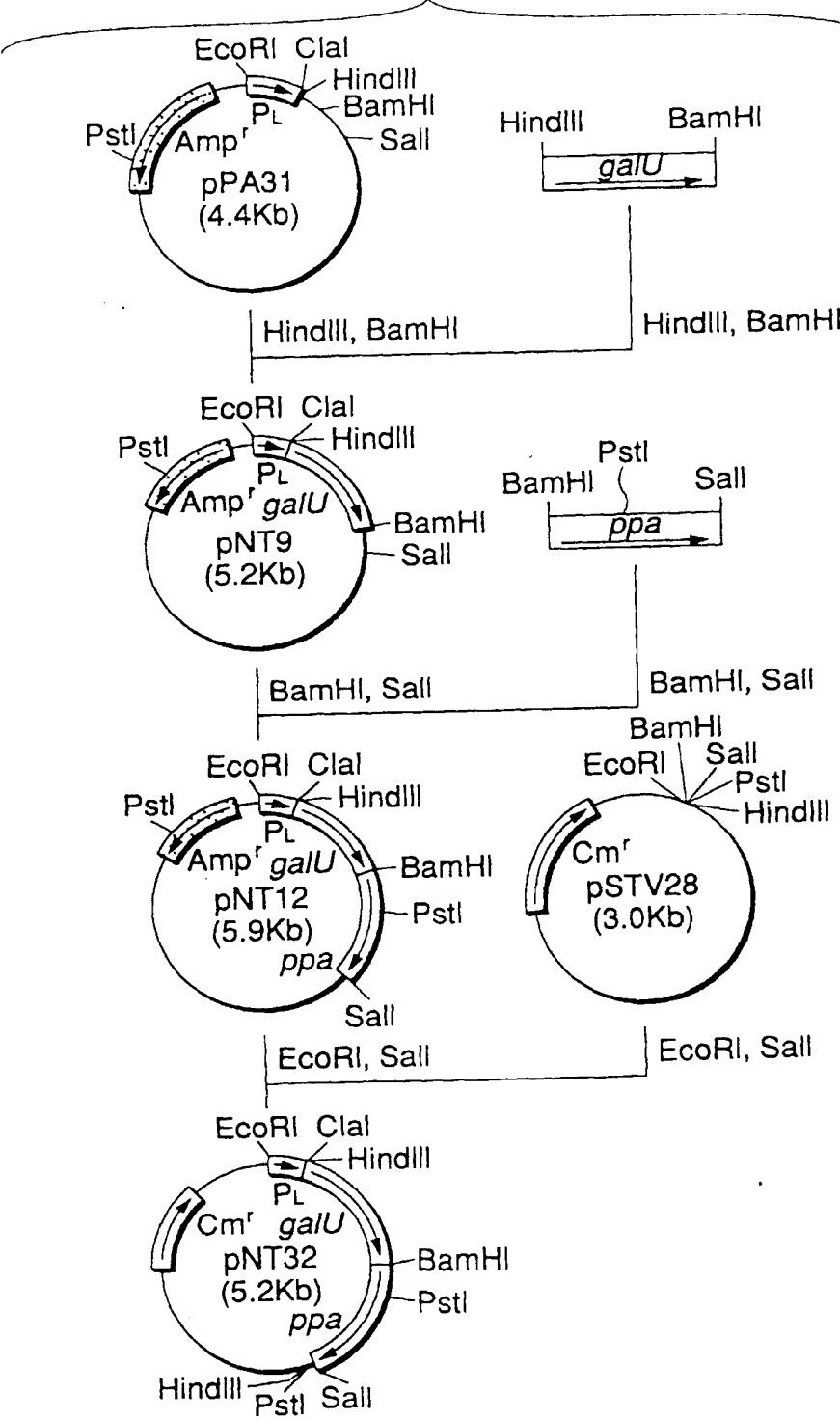


FIG.3

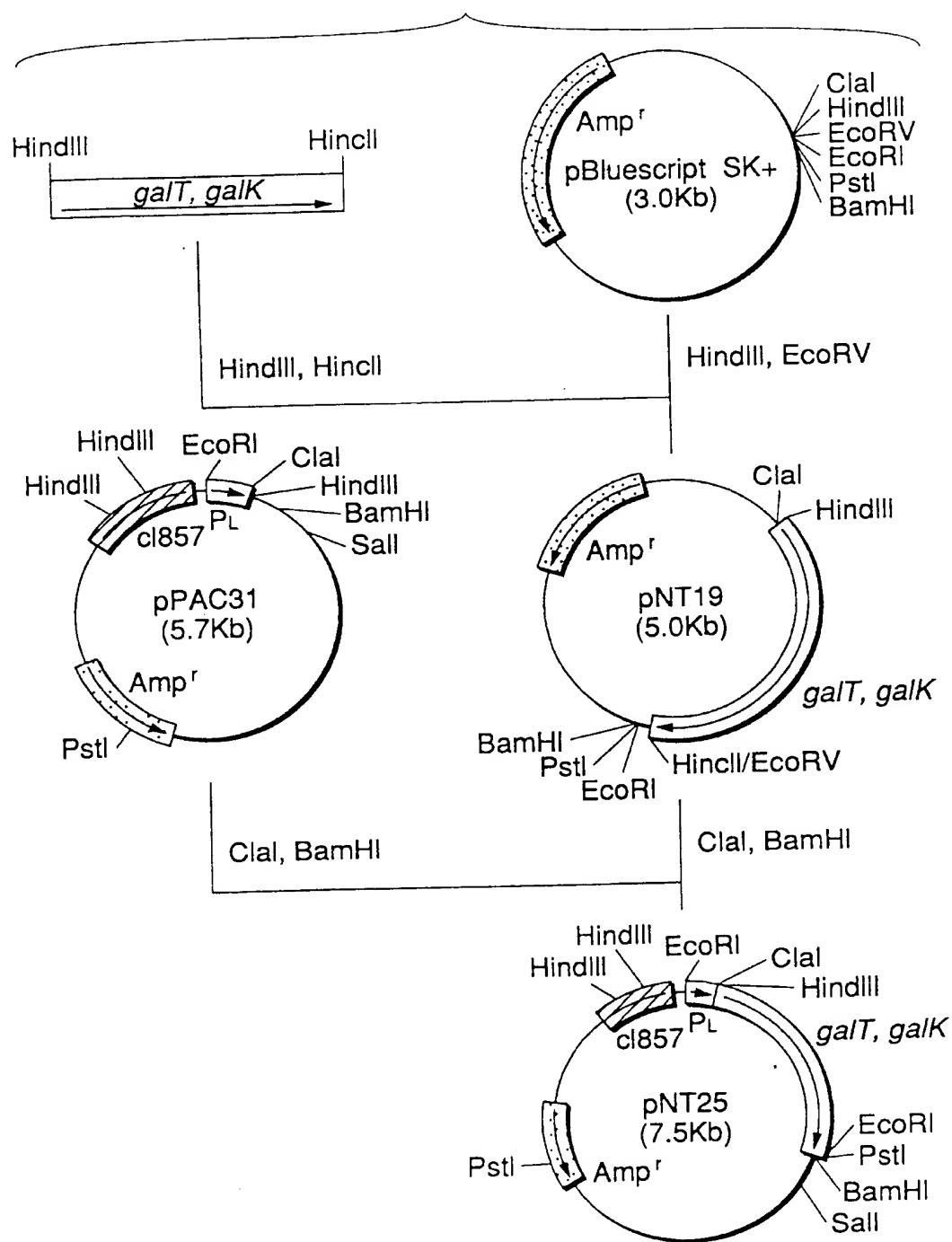


FIG.4

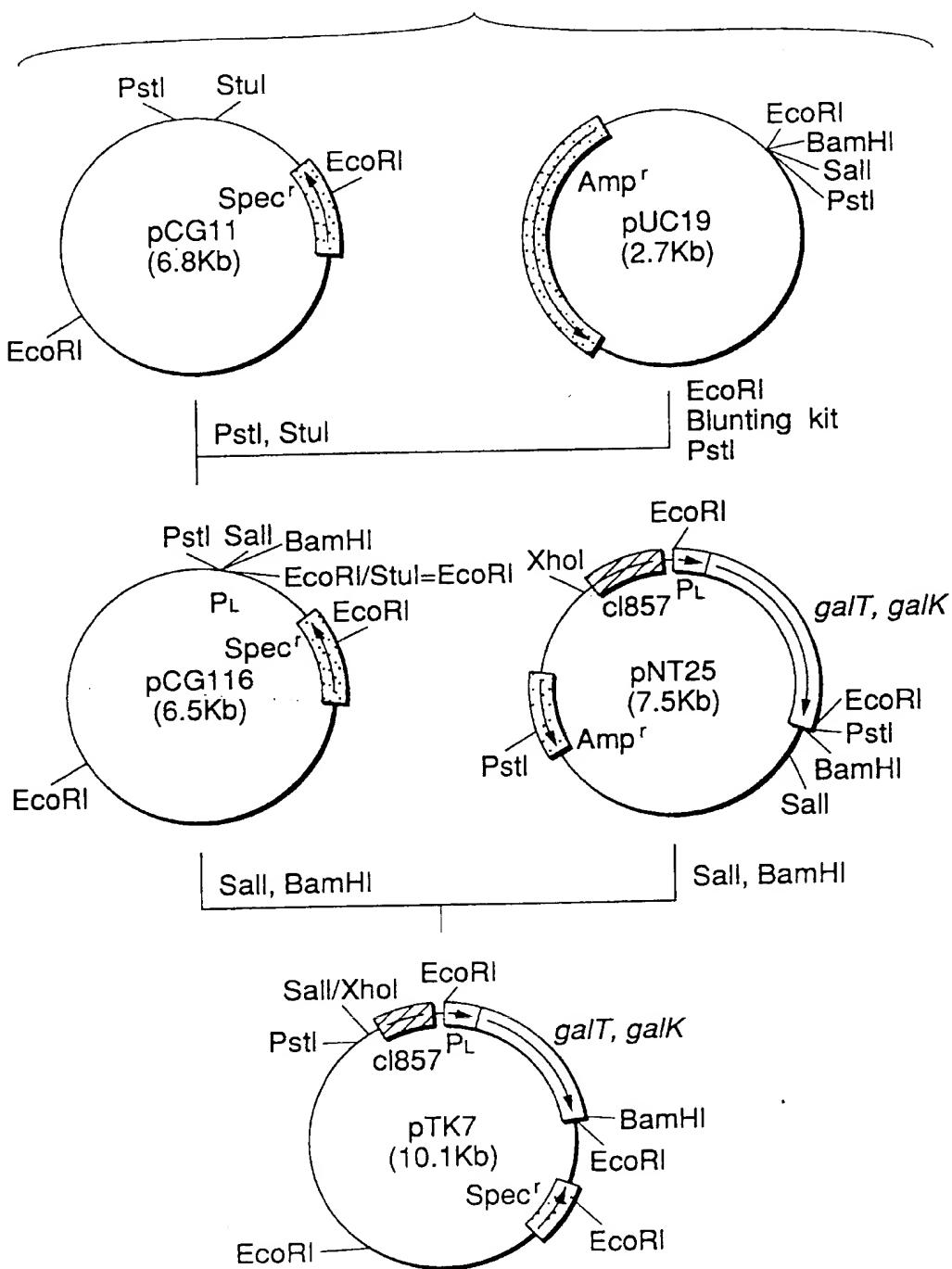


FIG.5

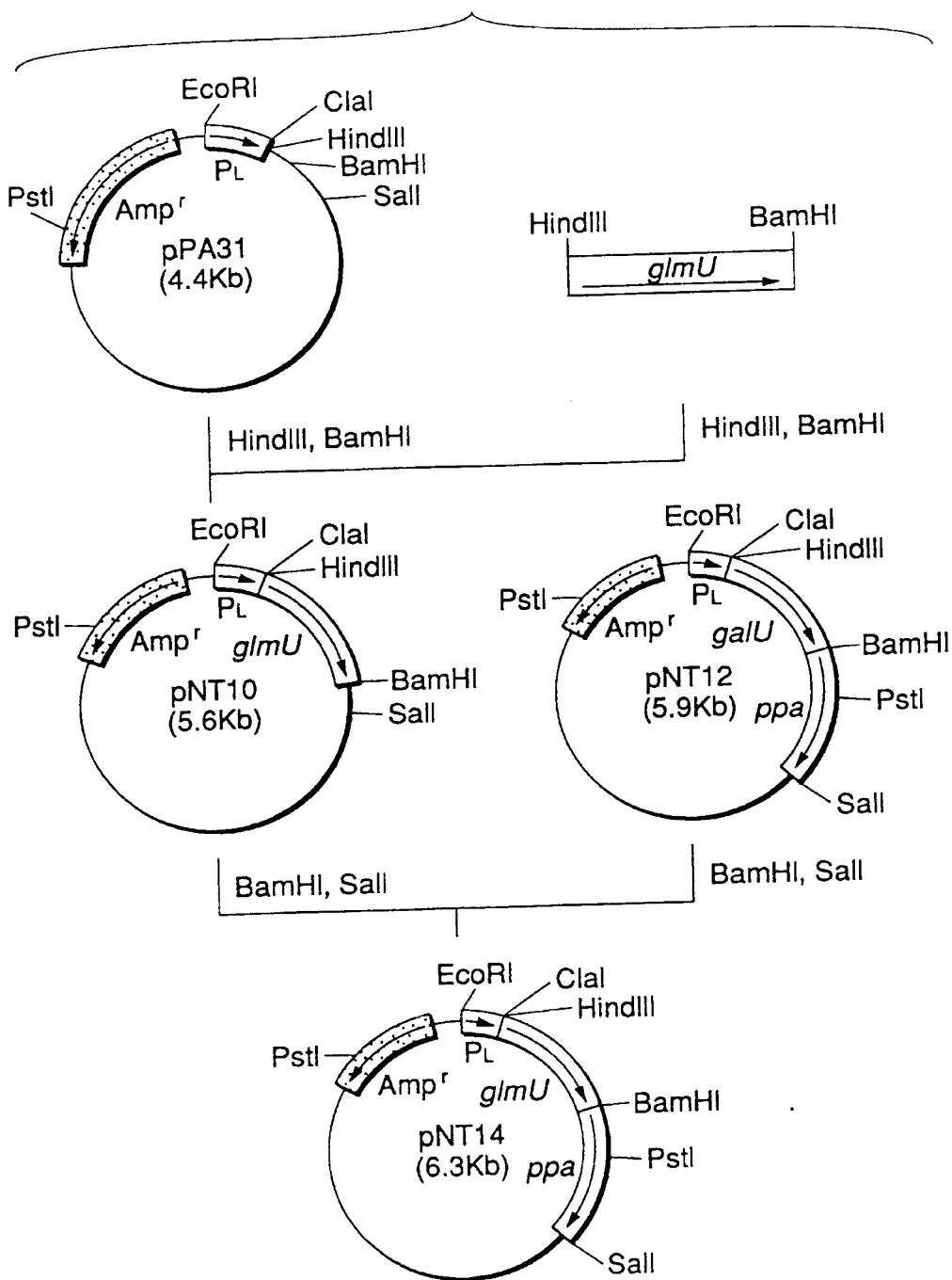


FIG.6

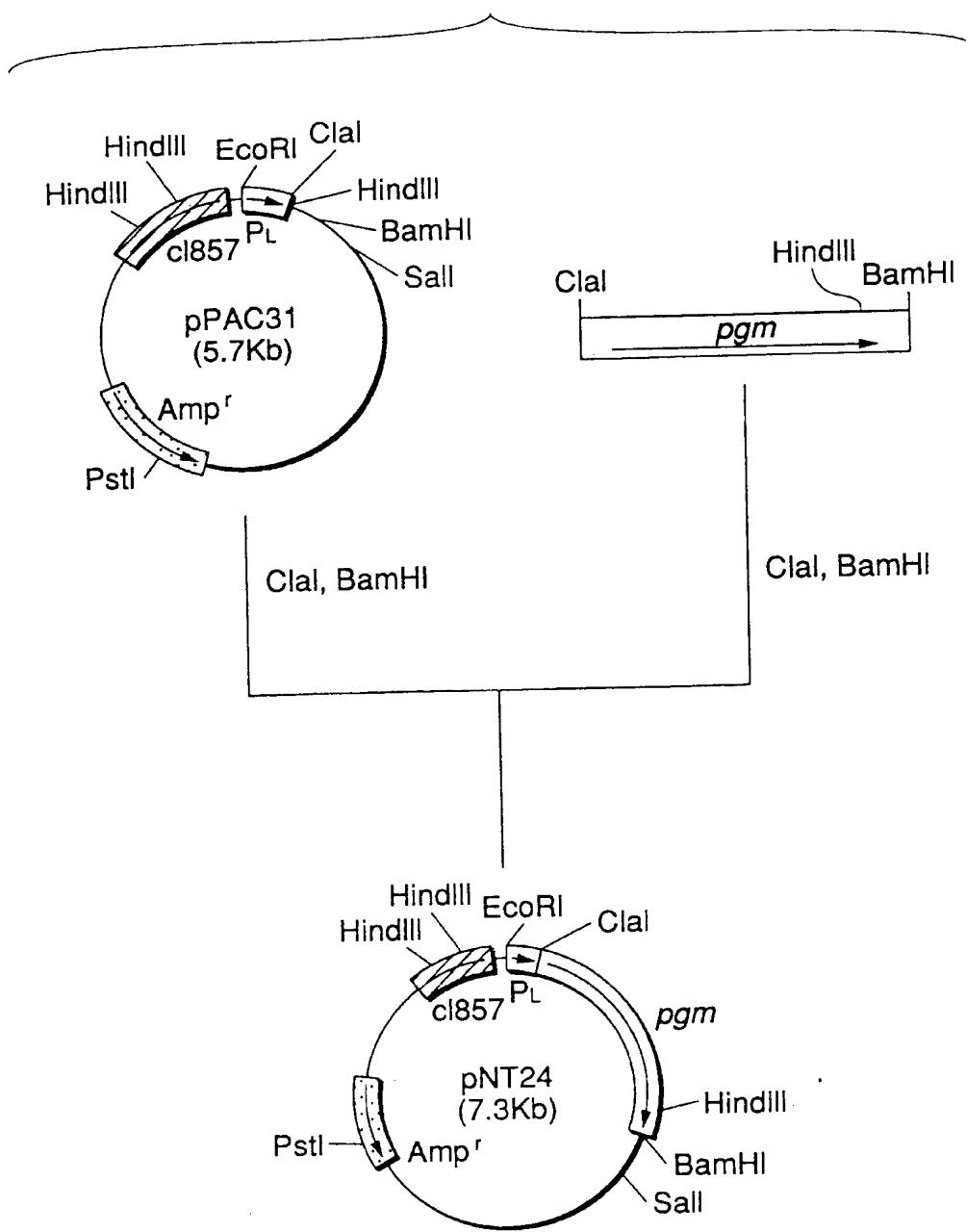


FIG.7

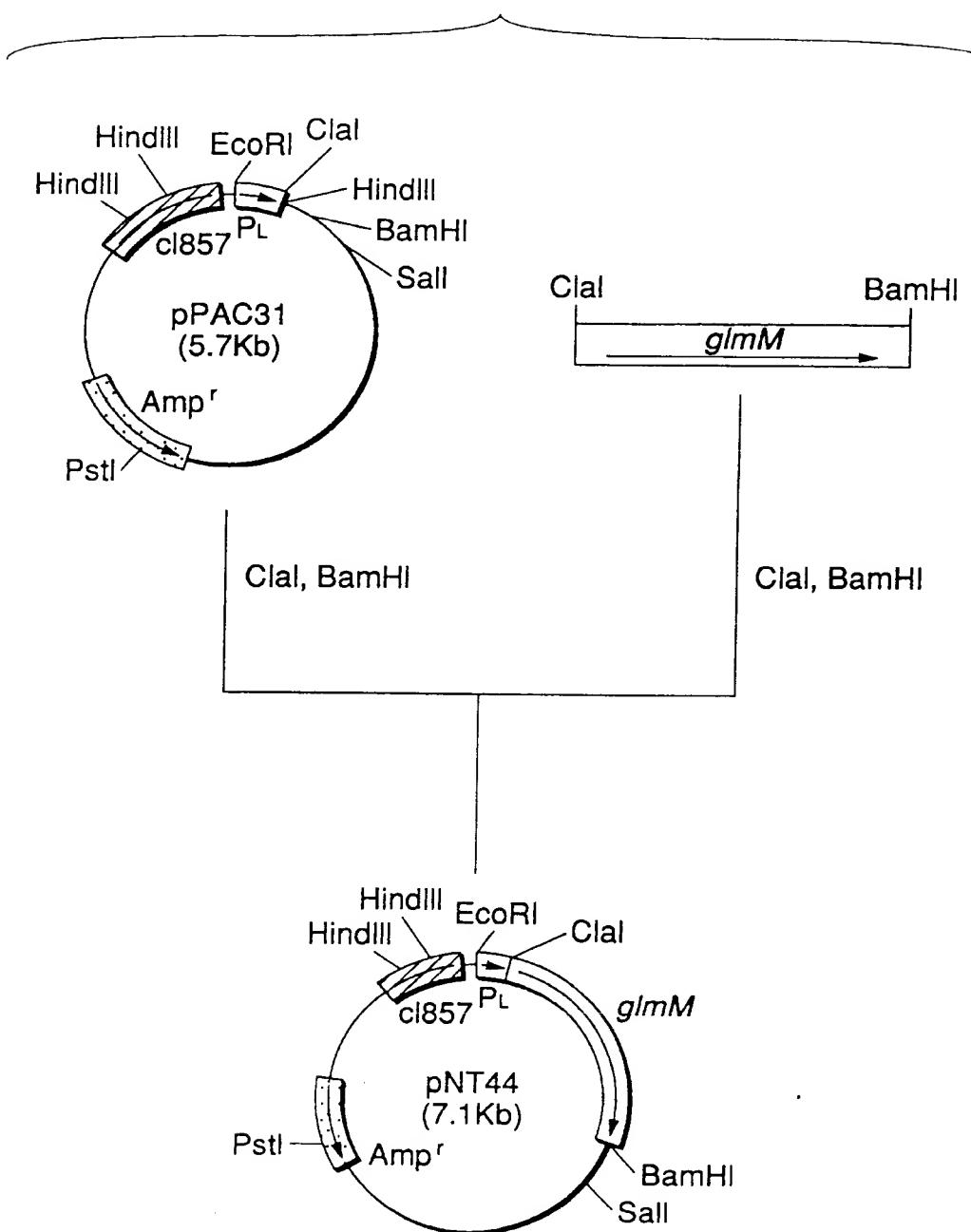


FIG.8

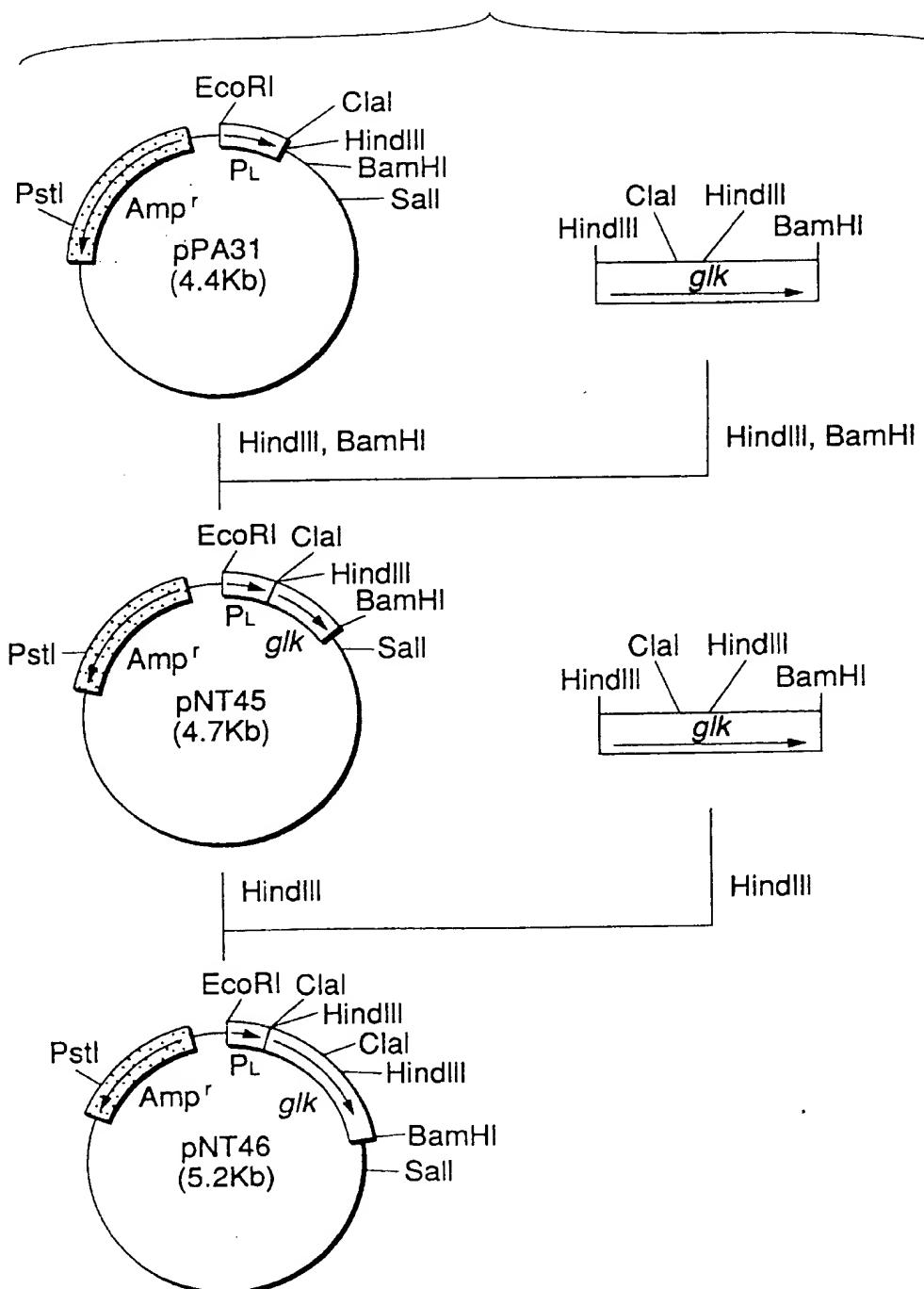


FIG.9

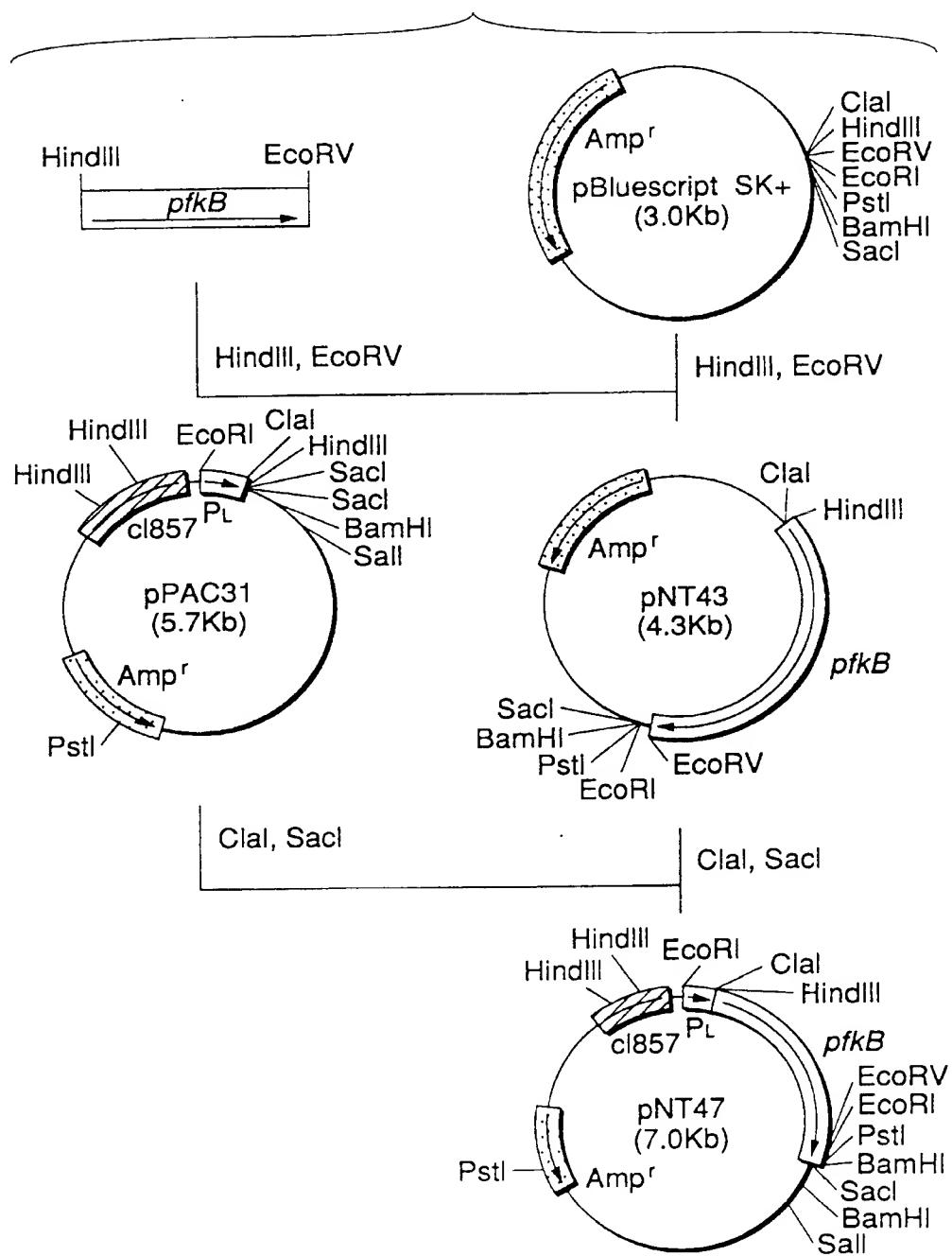


FIG.10

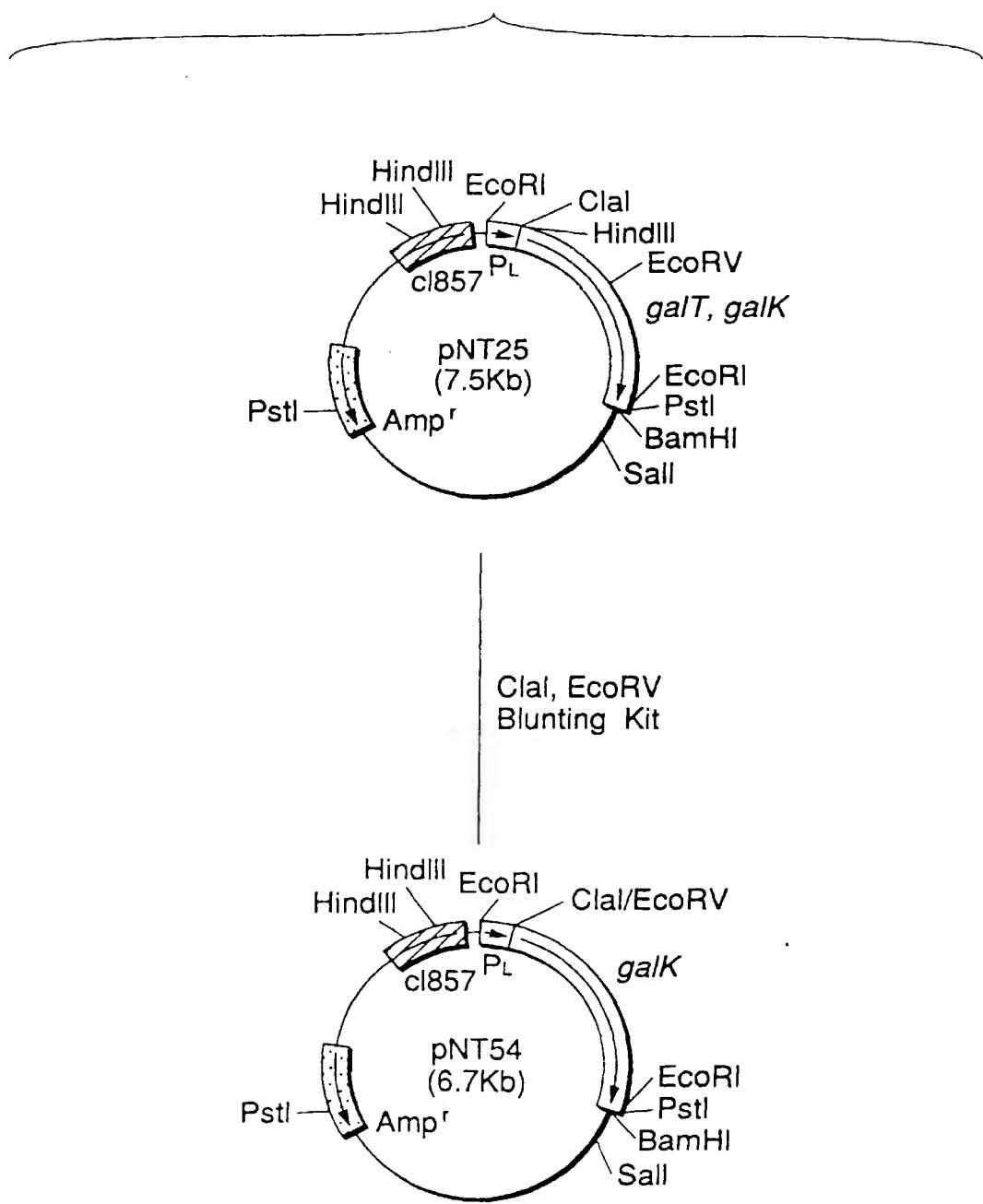


FIG.11

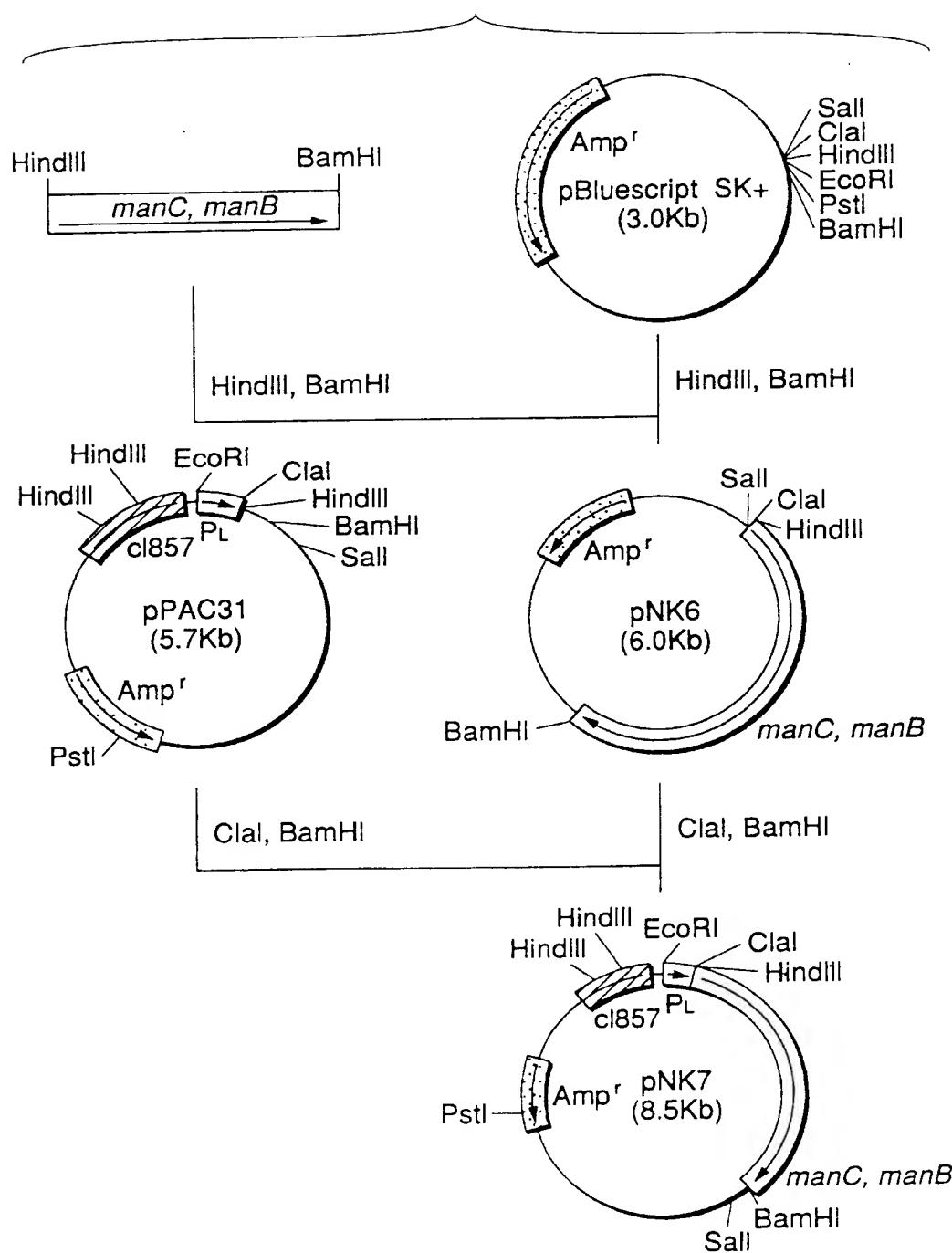


FIG.12

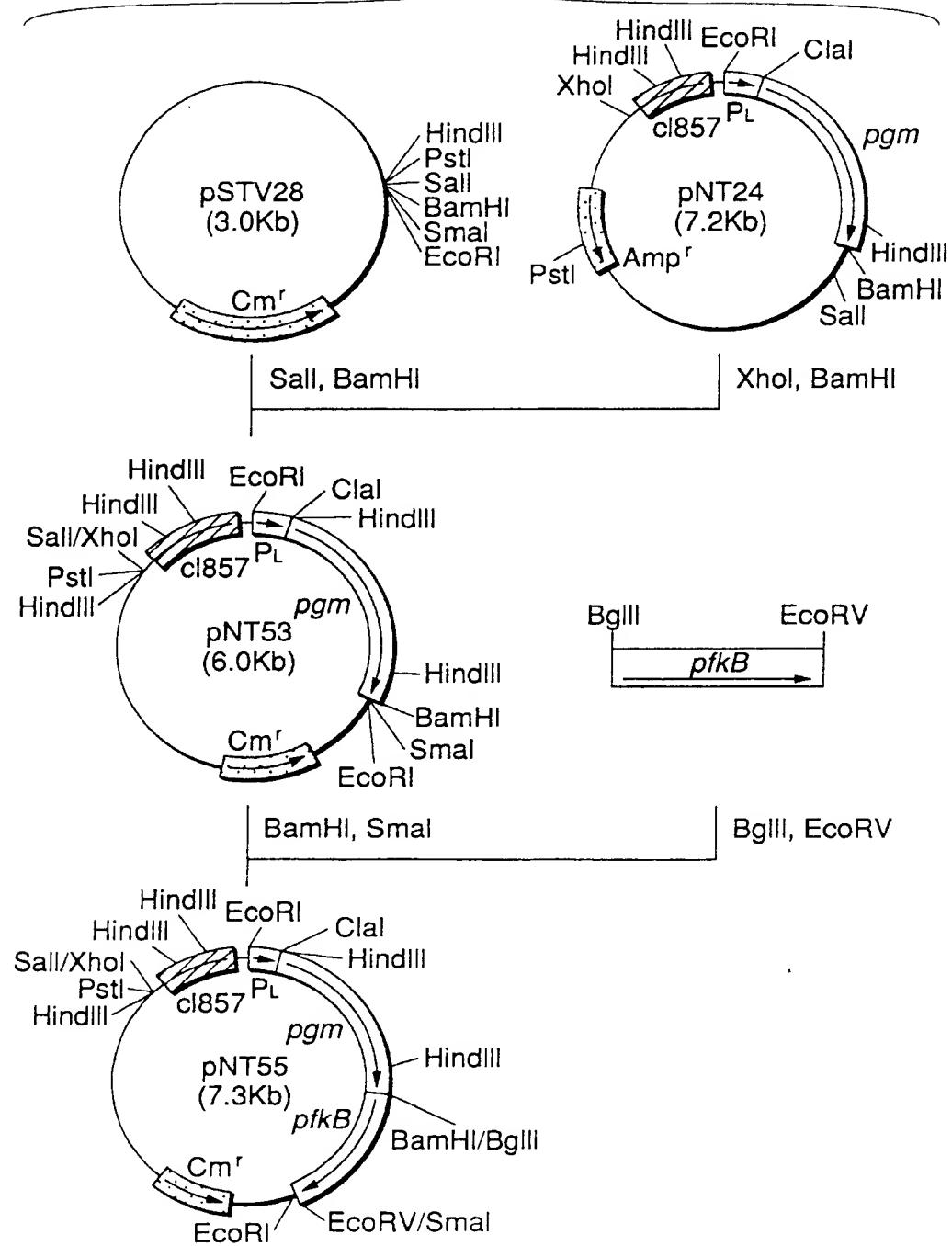


FIG.13

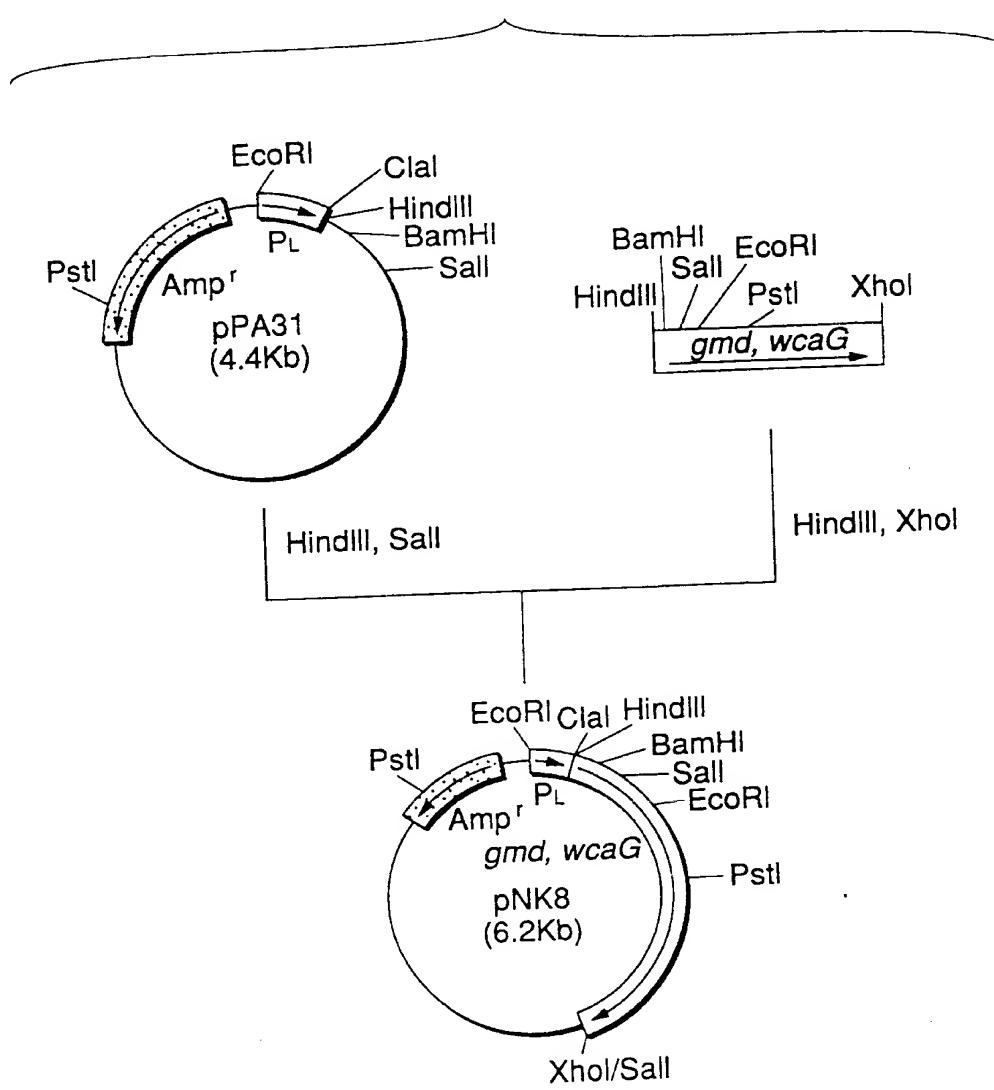


FIG. 14

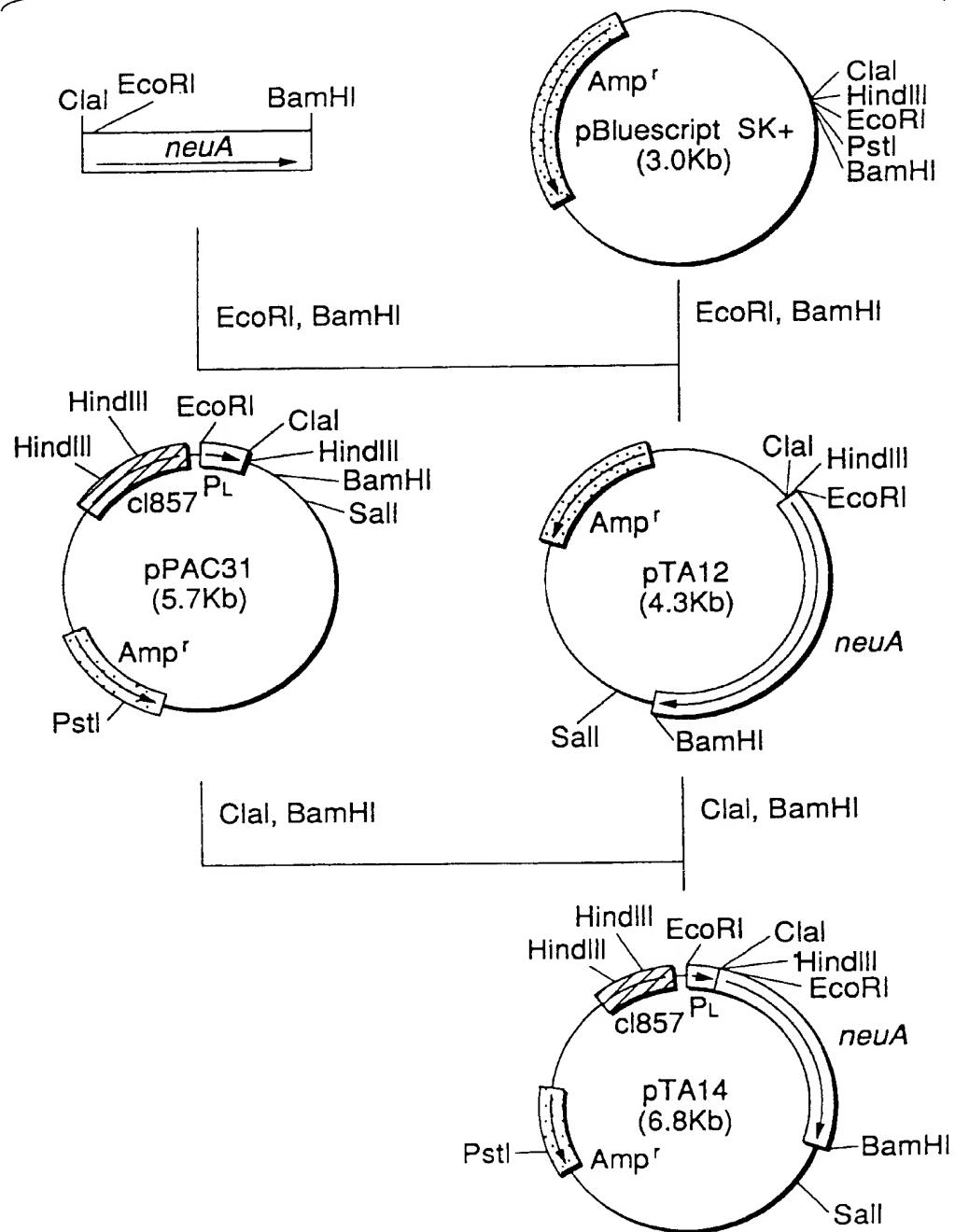


FIG.15

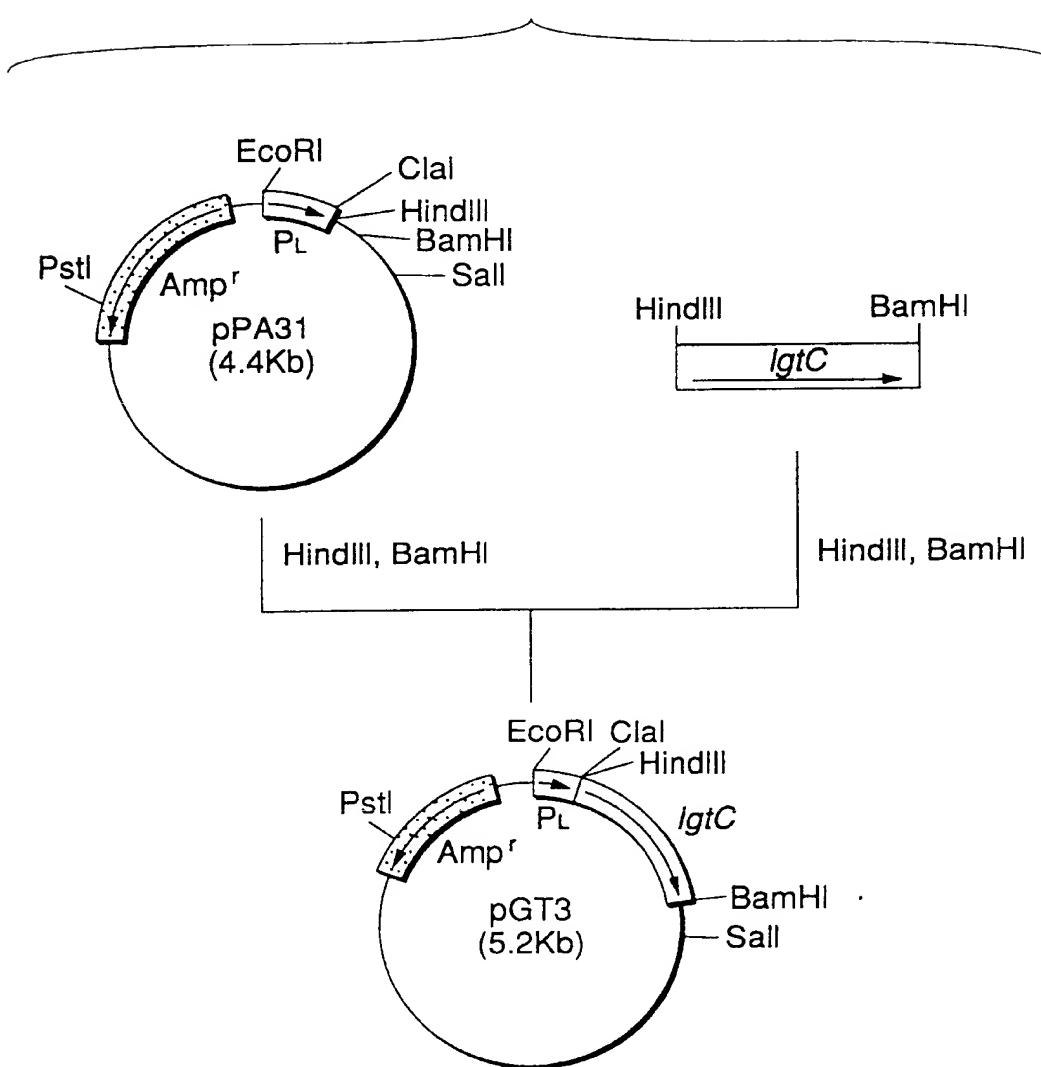
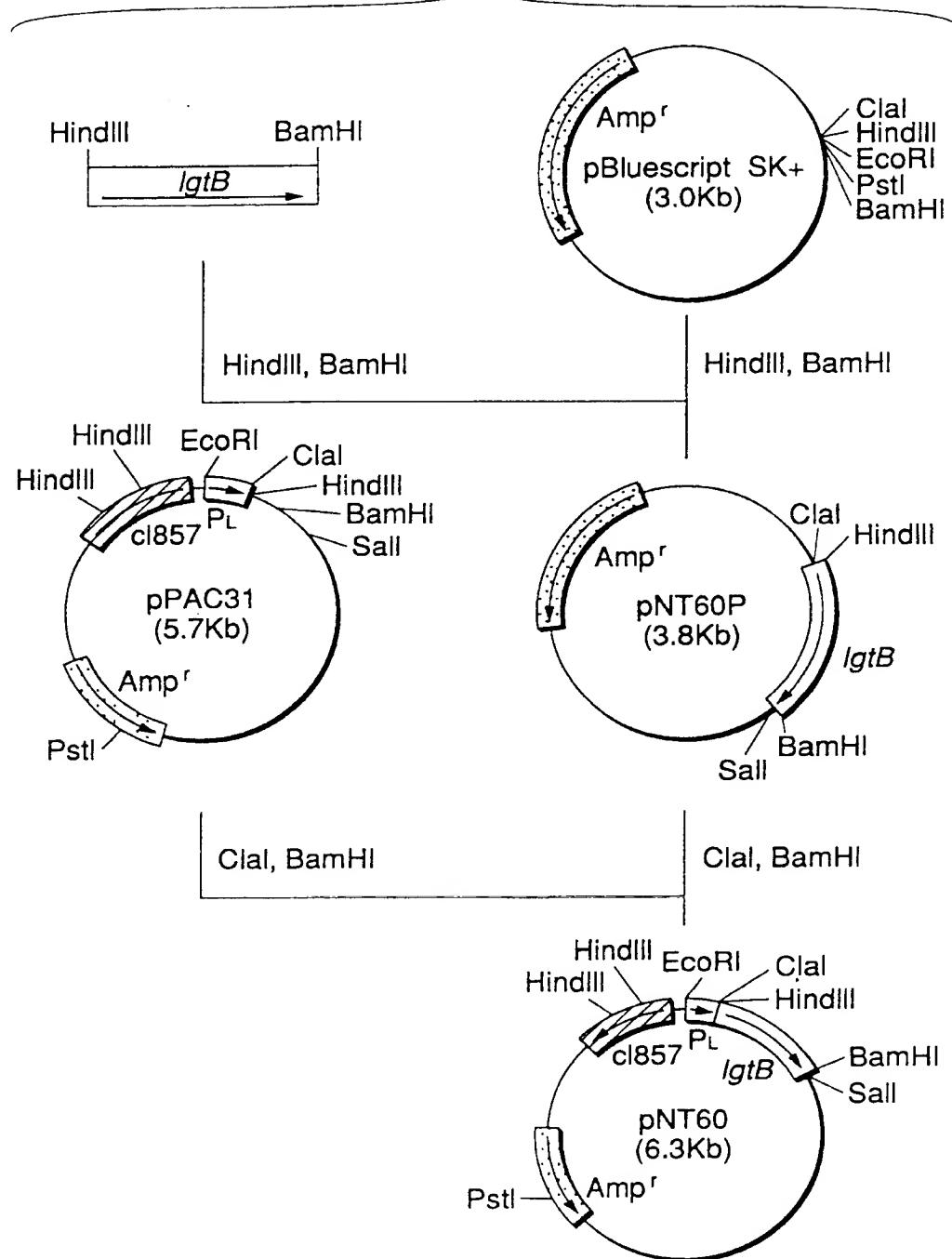


FIG.16



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/03226

**A. CLASSIFICATION OF SUBJECT MATTER**  
 Int. Cl<sup>6</sup> C12P19/26, C12N1/21, C12N15/54, C12N5/16 // (C12P19/26, C12R1:19, C12R1:15)

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl<sup>6</sup> C12P19/26, C12N1/21, C12N15/54, C12N5/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 WPI (DIALOG), BIOSYS (DIALOG), CA (STN), REGISTRY (STN)**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category <sup>a</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 47-1837, B1 (Koichi Ogata), January 19, 1972 (19. 01. 72) (Family: none)	1, 4-8 2-3, 9-61
X	JP, 46-40756, B1 (Koichi Ogata), December 1, 1971 (01. 12. 71) (Family: none)	1, 4-8 2-3, 9-61
X	JP, 62-134096, A (Snow Brand Milk Products Co., Ltd.), June 17, 1987 (17. 06. 87) (Family: none)	1, 4-8 2-3, 9-61
X	JP, 47-46351, B1 (Marukin Shoyu Co., Ltd.), November 22, 1972 (22. 11. 72) (Family: none)	1, 4-8 2-3, 9-61
X	JP, 57-18893, A (Asahi Chemical Industry Co., Ltd.), January 30, 1982 (30. 01. 82) (Family: none)	1, 4-8 2-3, 9-61
X	JP, 7-233187, A (Fuso Pharmaceutical Industries, Ltd.), September 5, 1995 (05. 09. 95) (Family: none)	1, 4-8 2-3, 9-61

Further documents are listed in the continuation of Box C.  See patent family annex.

- Special categories of cited documents:**
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search December 11, 1997 (11. 12. 97)	Date of mailing of the international search report January 13, 1998 (13. 01. 98)
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Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/03226

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 1-500560, A (Getty Sci. Dev. Co.), March 1, 1989 (01. 03. 89) & WO, 87/05937, A & NO, 179875, B & EP, 380470, B	15 - 61

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/JP97/03226

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The common matter in Claims 1, 3, 4, 5, 6-7, 8, 15-16, 17-20, 21-25, 26-39, 40-57, and 58-61 is a process for producing sugar nucleotides as set forth in Claim 1.

However, the search conducted has revealed that these processes are not novel ones but the ones disclosed in Reference 1 (JP, 47-1837, B (Koichi Ogata) Jan. 1, 1972 (19. 01. 72)), Reference 2 (JP, 46-40756, B (Koichi Ogata), Dec. 1, 1971 (01. 12. 71)) and Reference 3 (JP, 62-134096, A, Snow Brand Milk Products Co., Ltd.), June 17, 1987 (17. 06. 87)). (The enzyme source described in Claim 1, i.e., a culture of a microorganism, includes cells obtained by centrifuging the culture and an enzymatic preparation obtained by

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**  The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

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Continuation of Box No. II of continuation of first sheet (1)

extracting these cells after being mechanically disrupted, as described in Claim 4. It is stated in the references that the enzyme sources usable in References 1 and 2 and the microorganisms usable in Reference 3 involve not only the cells per se and cultures containing the cells, but also disrupted cells, cell extracts, etc. Thus, the enzyme source as described in Claim 1 corresponds to the enzyme sources in References 1 and 2 and the microorganisms in Reference 3. Accordingly, there is no difference among them.)

Consequently, the process for producing sugar nucleotides as claimed in Claim 1 does not lie outside the category of the prior art and, therefore, the common matter (the invention of Claim 1) is not a special technical feature under the provisions of the second sentence of Rule 13(2) of the Regulations under the PCT.

Therefore, there is no common matter in Claims 1, 3, 4, 5, 6-7, 8, 15-16, 17-20, 21-25, 26-39, 40-57, and 58-61. Since there is no common feature regarded as a special technical feature under the provisions of the second sentence of Rule 13(2) of the Regulations under the PCT, these inventions differing from each other are not technically linked to each other under the provisions of Rule 13 of the Regulations under the PCT. Such being the case, the inventions of Claims 1, 3, 4, 5, 6-7, 8, 15-16, 17-20, 21-25, 26-39, 40-57, and 58-61 do not comply with the requirement of unity of invention.